CHARACTERISATION OF SOME ASPECTS OF THE NEUTROPHIL ELASTASE:SERPIN BALANCE IN THE HORSE

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DECLARATION

This thesis has been composed by myself and the work contained herein is my own.

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LIST OF ABBREVIATIONS

AEC	3-amino-9-ethylcarbazole substrate
API	alpha-1-proteinase inhibitor
APS	ammonium persulphate
BALF	broncho-alveolar lavage fluid
BSA	bovine serum albumin
CHO	carbohydrate
COPD	chronic obstructive pulmonary disease
Cy3	cyanine 3
DAB	3,3' -diaminobenzidine tetrahydrachloride
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N,-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENE	equine neutrophil elastase
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
His	histidine
HNE	human neutrophil elastase
HRPO	horse radish peroxidase
Ig	immunoglobulin
IL	interleukin
k_{ass}	association constant

kDa	kilodaltons
LRT	lower respiratory tract
LPS	lipopolysaccharide
mAb	monoclonal antibody
MOPC	mineral oil plasmacytoma
MPBS	dried skimmed milk in PBS
M_r	relative molecular weight
MW	molecular weight
MWCO	molecular weight cut off
NE	neutrophil elastase
NGS	normal goat serum
NSS	normal sheep serum
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	PBS+0.05% $Tween_{20}$
PBST80	PBS+0.5% $Tween_{80}$
PEG	polyethylene glycol
PELF	pulmonary epithelial lining fluid
PNGase F	peptide N-Glycosidase F
RSL	reactive site loop
RZ	protein to enzyme ratio
SDS	sodium dodecylsulphate

SEC	serpin enzyme complex
Ser	serine
SERPIN	serine proteinase inhibitor
Spi	serine proteinase inhibitor
Tb	Thoroughbred
Temed	N,N,N',N'-Tetramethylethylenediamine
TESPA	3-aminopropyltriethoxysilane
TMB	3,3',5,5'-tetramethylbenzidine

ABSTRACT

Pulmonary emphysema in the horse in response to lung disorders associated with an influx of neutrophils, such as chronic obstructive pulmonary disease (COPD), results in a restricted peribronchiolar pattern of tissue damage. Humans, in contrast, have a more extensive centrilobular pattern of emphysema in similar neutrophil associated lung disorders. The major cause of pulmonary emphysema has been shown to be excessive or unregulated neutrophil elastase activity which has led to the development of the 'proteinase:anti-proteinase theory of lung disease'. To evaluate the proteinase:antiproteinase balance in the horse in order to compare it to that found for human API some aspects of equine neutrophil elastase (ENE) and its main regulator in the lower respiratory tract, equine alpha-1-proteinase inhibitor (API), have been investigated.

Mouse monoclonal and sheep polyclonal antibodies specific to equine API were raised and used to show by immunohistochemistry that the distribution of equine API was very similar to that of the previously known distribution for human API. The major difference between the two species was that equine hepatocytes and bile ductules showed extensive and often intense positive staining for equine API which has not been demonstrated in equivalent human tissues.

Sheep polyclonal antibodies specific to ENE 2A, the most abundant ENE found in equine neutrophils, were raised and used to show that ENE 2A had a granular

distribution confined to the cytoplasm of neutrophils and that each neutrophil contained 0.813 ± 0.054 pg (mean ± SEM) of ENE2A. In contrast equine API had a uniform non-granular cytoplasmic distribution and each neutrophil contained 0.021 ± 0.003 pg of equine API.

In dynamic studies of Percoll purified equine peripheral blood neutrophils a positive correlation existed between the total superoxide anions (SOA) generated and release of ENE 2A when stimulated with zymosan activated serum over 90 minutes. These kinetic data suggest that SOA generation may have the potential to prolong the activity of ENE 2A in the neutrophil's immediate microenvironment by promoting oxidative inactivation of API thereby enhancing the neutrophil's contribution to local host tissue damage. Both these secretory mechanisms were dependent upon extracellular cations.

These findings suggest that the difference in anatomical distribution of pulmonary emphysema in horses and humans is probably due to a higher concentration of plasma derived equine API in pulmonary epithelial lining fluid. This, combined with the presence of oxidation resistant serine proteinase inhibitor (Spi) proteins, creates a more efficient antiproteinase screen in the neutrophil micro-environment in equine pulmonary tissues resulting in more effective inhibition of unwanted neutrophil elastase activity and subsequent host tissue damage.

Chapter 1

GENERAL INTRODUCTION

The ability of serum to inhibit proteolysis was noted over a century ago by Camus and Gley in 1897; they noticed that canine serum decreased the activities of pepsin and trypsin on milk proteins and albumin. However, it was the discovery of a link between a serum dysproteinaemia, unregulated neutrophil elastase activity and the development of pulmonary emphysema in humans (Laurell and Eriksson, 1963; Eriksson, 1964; Janoff and Scherer, 1968; Hayes *et al* 1975) that stimulated more intense medical research into proteolytic enzymes and their inhibitors.

1.1 Functional Importance of Proteolytic Enzymes

Protein metabolism and its regulation is one of the fundamental properties necessary for the maintenance of life. Proteolytic enzymes and their regulators are vital for digestion, blood coagulation, complement activation, host defence and a vast array of other processes (Bieth, 1986; Potempa *et al* 1994). The various specificities, rates and durations of these processes are all determined by proteolytic enzymes which are categorised broadly into 4 classes dependent upon their active site structure: serine, cysteine, aspartic and metallo-proteinases (Neurath, 1996) and their respective inhibitors. The name of each group refers to the key amino acid residue or component (i.e. zinc in metallo-proteinases) of the active site of the enzyme which dictates enzyme activity and specificity. The active site, which usually lies in a cleft on the surface of the molecule, consists of a unique spatial arrangement of amino acid

residues which confer on the enzyme the ability to hydrolyse peptide bonds of certain amino acid sequences. The nomenclature of the subsites varies about the named catalytic group (e.g. serine, cysteine etc.) such that they are numbered S_1, S_2, etc. away from the catalytic site towards the N-terminus and S_1', S_2' etc. towards the C-terminus. The corresponding alignment on any peptide substrate is denoted by P, hence S_1 aligns with P_1, S_2 with P_2 and so forth (Barrett, 1986). The conformation of the whole enzyme depicts whether the active site can be brought into close association with these peptides both physically and electrostatically. In this way the substrate specificity of an enzyme is determined.

1.2 Serine Proteinases

The serine proteinases are the most studied family of mammalian proteolytic enzymes and probably the most evolutionary successful as they are the most numerous and diverse group (Barrett, 1986). Historically, studies began with the digestive enzymes of the pancreas (especially trypsin and pancreatic elastase) because of the relative ease of obtaining reasonable quantities of the enzymes and the direct application of the findings to an observable process such as digestion. The active site of serine proteinases is found typically on a surface depression rather than in a cleft like the other families of proteinases (Fersht, 1985).

1.2.1 Mechanism of Action

Peptide and synthetic ester substrates are hydrolysed by the acyl-enzyme mechanism.

The enzyme and substrate initially form a non-covalent enzyme-substrate complex due to spatial conformity and physical forces of attraction in the enzyme's specificity pocket (i.e. electrostatic, Van der Waals, etc.) (Branden and Tooze, 1991). The serine-195 hydroxyl group of the proteinase which has an induced negative charge then mounts a neucleophilic attack on the induced positively charged carbonyl carbon atom of the substrate. This is catalysed by the histidine-57 residue acting as a general base and results in a tetrahedral intermediate which in turn becomes an imidazolium ion which then breaks down by acid catalysis to an acyl-enzyme intermediate and amine product (first peptide product). The proton of the serine hydroxyl group is transferred to the leaving amine group. The acyl-enzyme is then hydrolysed by the reverse reaction pathway of acylation, however, a water molecule instead of the serine residue is now the attacking nucleophile, this allows release of the second peptide product and regeneration of enzymatic activity (Fersht, 1985; Barrett, 1986; Polgar, 1990).

1.3 Human Neutrophil Elastase

Human neutrophil elastase (HNE) is the most studied non-digestive mammalian serine proteinase due to its role in several well documented pathological conditions (Janoff, 1985b). Research has revealed that neutrophil elastase (NE) is essential for normal host defence and the advantages of its presence far outweigh those of its absence (Dale *et al* 1972).

A proteinase may only be called an 'elastase' if it is capable of solubilising mature

cross-linked elastin as HNE does, but this does not imply that elastases are specific for elastin as they hydrolyse many other proteins (Bieth, 1986). Although the most abundant form of non-pancreatic elastase is neutrophil elastase, elastases have also been found in peripheral blood monocytes and alveolar macrophages and are collectively referred to as leukocyte elastases (Bieth, 1986; Campbell *et al* 1989). The first pathological process HNE was associated with was vascular endothelial damage (Janoff and Scherer, 1968). The link between its excessive and/or unregulated activity with pulmonary emphysema was not confirmed experimentally until much later (Hayes *et al* 1975; Janoff *et al* 1977).

1.3.1 Structure

Human neutrophil elastase is a 29kDa, 218 amino acid, single chain globular glycoprotein stabilised by 4 disulphide bonds linking eight half cysteine residues (Sinha *et al* 1987; Crystal, 1996). The presence of 19 arginine residues, which considerably outweigh the properties of the eight acidic residues, are responsible for the highly basic nature of HNE (Crystal, 1996). All but one of the arginine residues are located on the surface of the molecule in a horse-shoe like manner around the active site. This arrangement contributes to the enzymes preference for binding to linear sulphated polysaccharides and to the negatively charged proteoglycan matrix of the neutrophil storage granule (Crystal, 1996). Two asparagine linked carbohydrate (CHO) side chains (Asn-95 and Asn-144) are present and variations in these account for the three or four isoforms of HNE found on gel electrophoresis in some studies (Baugh and Travis, 1976; Vanhalbeek *et al* 1988; Watorek *et al* 1993). Both CHO

side chains point away from the active site and into the surrounding milieu and so are unlikely to interfere with the binding of smaller protein substrates or inhibitors, however, they may become entangled within the extended elastin network of the extracellular matrix (Crystal, 1996).

1.3.2 Mechanisms of Elastase Release From Neutrophils

Active release of intracellular stores of HNE from neutrophils occurs by two main pathways; (1) the release by fusion of azurophilic storage granules with plasmalemma which is committed to, or has already formed, phagocytic vacuoles and (2) phagocytosis independent release of azurophilic storage granule contents by fusion with areas of the cell plasma membrane that are not involved in phagocytic uptake (reviewed in Baggiolini and Dewald, 1984). Passive release of HNE occurs due to cell death and disintegration (necrosis) of the neutrophil usually at sites of infection. Neutrophil elastase is also found in small amounts in close association with the cell membrane facilitating diapedesis (Henson and Johnston, 1987).

1.3.3 Gene Expression and Protein Localisation

The HNE gene has been located on chromosome 11 at q14 and consists of 5 exons and 3 introns (Takahashi *et al* 1988b) and is only expressed in bone marrow myelocytic precursor cells (Takahashi *et al* 1988a), especially promyelocytes (Fouret *et al* 1989). Expression of the gene is regulated primarily at the level of gene transcription (Yoshimura and Crystal, 1992). The coding exons of the NE gene predict a primary translation product of 267 residues which includes 29 residue N-

terminal and 20 residue C-terminal precursor peptides. Further analysis of the N-terminal peptide sequence reveals a 27 residue 'pre' signal peptide and a 'pro_N' dipeptide, similar to that of other blood cell lysosomal proteases (Takahashi *et al* 1988b). This 'pro_N' peptide would allow intracellular transport and temporary storage prior to being packaged into the primary (azurophilic) granules of the cytoplasm of neutrophils without the risk of intracellular proteolysis (Crystal, 1996). In normal adults each mature neutrophil contains about 1.1pg of HNE (Campbell *et al* 1989) stored in the primary cytoplasmic granules (Dewald *et al* 1975; Cramer *et al* 1989).

1.3.4 Physiological Functions

Human neutrophil elastase is a neutral protease, with a very catholic consumption in terms of substrates, which can be rapidly transported to any part of the body via the highly mobile peripheral blood neutrophil in response to chemotactic inflammatory mediators (Bieth, 1986). This mobility is greatly dependent upon HNE itself as it is involved in neutrophil migration through vascular endothelium to gain access to extra-vascular compartments (Henson and Johnston, 1987). Large amounts of elastase can be rapidly recruited into areas of inflammation to assist host defence and in remodelling of damaged tissues through its ability to cleave many components of the extracellular matrix. Platelet activation (Selak, 1992), oxygen independent bactericidal activity (Ganz *et al* 1987) and digestion of micro-organisms and other material within the phagolysosome (Janoff, 1985b) also require elastase activity (Janoff and Scherer, 1968; Mainardi *et al* 1980; Heck *et al* 1985; Sinha *et al* 1987; Bieth, 1986). Much of HNE role in host defence has been elucidated through the study of Chediak-Higashi

syndrome, a rare autosomal recessive condition of humans where the neutrophils are deficient in NE resulting in increased susceptibility to pyogenic infections (Dale *et al* 1972; Ganz *et al* 1988).

1.3.5 Regulation of HNE Activity

In the healthy state, the activities of HNE in the execution of host defence and tissue remodelling are precisely controlled. Extracellular and intracellular HNE is regulated by serine proteinase inhibitors such as alpha-1-proteinase inhibitor (API) (Gadek *et al* 1981b; duBois *et al* 1991; Mason *et al* 1991) and secretory leukocyte proteinase inhibitor (SLPI) (Sallenave *et al* 1997a). Similarly, α2-macroglobulin, a plasma protein of high molecular weight which can inhibit proteinases from many different families, plays a role in the regulation of HNE (Travis and Salvesen, 1983). In addition to inhibition of free elastase, phagocytosis of apoptotic neutrophils protects tissues from the potentially harmful consequences of exposure to the contents of effete cells (Savill *et al* 1993). Activated cells of the monocyte/macrophage cell line are the main phagocytes involved in this process but other cells such as glomerular mesangial cells have also been shown to phagocytose apoptotic neutrophils (Duvall *et al* 1985; Savill *et al* 1989; Savill *et al* 1992).

1.3.6 Pathology Associated With Neutrophil Elastase

Due to the extensive range of substrates which HNE hydrolyses (Bieth, 1986), any prolonged breakdown in regulation will ultimately lead to unwanted host tissue damage. It has been shown that a so-called marginated pool of neutrophils exists in

the lung, which is in a dynamic equilibrium with the circulating pool, and is released into the main circulation when cardiac output is increased by adrenaline and is conserved when output subsequently falls (reviewed in Rossi and Hellewell, 1994). This suggests that the lung plays a significant role in neutrophil kinetics despite containing tissues which are highly sensitive to the action of its elastinolytic enzymes. The sequestration of large numbers of neutrophils in the pulmonary circulation is probably a response to the susceptibility for microbial invasion of the large surface area of the lungs vital to gaseous exchange. However, this large pool of neutrophils means that the lung is also highly susceptible to host induced tissue damage and emphysema.

Pulmonary emphysema in humans is predominantly associated with cigarette smoking and the accumulation and degranulation of neutrophils in the lung. This may be the result of 'frustrated phagocytosis' in the neutrophils and specific activation of them, either directly from inhaled smoke particles or via inflammatory mediators (Travis *et al* 1994; Gadek and Pacht, 1996a). Some of these inflammatory mediators are released by alveolar macrophages which make up the largest percentage of resident cells in the airways and contain a metalloelastase which is also capable of solubilising mature cross-linked elastin (Snider *et al* 1991). Human NE has been immunolocalised adjacent to elastin fibres in emphysematous lungs but not in samples of normal pulmonary tissue (Damiano *et al* 1986) suggesting that it is a major factor in the pathogenesis of the disease.

Other diseases in which HNE has been shown to play a significant pathogenic role are cystic fibrosis (Suter *et al* 1986; Suter and Chevallier, 1991), adult respiratory distress syndrome (Gadek and Pacht, 1996b), glomerulonephritis (Stauber *et al* 1994), vascular endothelial damage (Smedly *et al* 1986) and rheumatoid arthritis (Roughley and Barrett, 1977; Travis *et al* 1980; Matheson *et al* 1982).

Failure of HNE regulation can come about by two basic mechanisms; a fault in the normal proteinase inhibitory function (API deficiency, Perlmutter, 1996, see below), or overwhelming of the normal antiproteinase screen by excessive proteolytic activity. These two mechanisms have led to the delicate balance between these two factors being referred to as the 'proteinase:antiproteinase theory' of disease (Shapiro, 1995).

1.4 Equine Neutrophil Elastase

Initial studies of equine neutrophil contents found three proteinases present with elastinolytic properties which were named Proteinase 1, 2A and 2B (Dubin *et al* 1976; Potempa, 1982). Subsequent studies, including those in our own laboratory have, however, failed to support these findings as only two proteinases which will be referred to as equine neutrophil elastase (ENE) 2A and 2B have been purified from equine neutrophils (Koj *et al* 1976; Scudamore *et al* 1993; Pemberton *et al* 1993; Dubin *et al* 1994).

The amount of total ENE present, as measured by enzyme activity, has been previously estimated as 0.4pg/equine neutrophil in a ratio of 3:1 ENE 2A:ENE 2B

respectively (Von Fellenberg *et al* 1985; Dubin and Koj, 1986). This suggests that the amount of elastase present in horse neutrophils is a little over a third that in human neutrophils (Campbell *et al* 1989).

1.4.1 Structure and Biochemical Properties

Both equine elastases are neutral proteases and their biochemical properties are summarised in Table 1.1. The structure of neither protein has been fully elucidated but amino acid sequencing of the N-terminus, including the substrate binding regions, indicates that the two elastases show a 71% sequence identity to each other. When compared to HNE, ENE2A and ENE2B show a 68% and 86% sequence identity respectively. Within the first 34 amino acid residues of the N-terminus ENE2B differs from HNE in only three positions (Dubin *et al* 1994).

Property	Elastase 2A	Elastase 2B	Human Elastase
Relative molecular weight (M_r)	24500	20500	29000
Polypeptide chains	1	1	1
Isoenzymes	1	1	3
Isoelectric point pI	8.8	>10	>9
pH optimum of activity			
a) casein	7.4	7.4	7.0
b) elastin	7-10	7	
Active centre	Ser-His	Ser-His	Ser-His

Table 1.1 - Properties of elastases from horse and human leukocytes. Pooled data from Dewald *et al* 1975; Dubin *et al* 1976; Baugh and Travis, 1976; Bieth, 1986; Sinha *et al* 1987; Watorek *et al* 1993.

Both forms of equine elastase have identical specificity when exposed to the oxidised insulin B-chain by preferentially attacking the peptide bonds with small aliphatic

amino acids (valine and alanine) at their P1 site (Dubin *et al* 1994). Despite this, ENE 2B displays a seven fold higher elastinolytic activity than HNE, which has a similar elastinolytic activity to that of ENE 2A (Potempa *et al* 1985). These differences probably stem from amino acid residue variations in the primary structure causing alterations to spatial conformity between whole elastase and elastin molecules, and to electrostatic forces on secondary binding sites and on the active sites on the three respective enzymes. Thus, although the amino acid residues around the reactive site are critical for determination of substrate specificity and kinetics of inhibition they are moderated to some extent by the overall tertiary structure of the enzyme (Dubin *et al* 1994).

1.4.2 Physiological Functions and Pathology

Direct knowledge of the physiological and pathological roles of equine elastases has not been documented therefore all assumptions about their roles have been made by analogy with human data (Dubin *et al* 1994). With respect to disease, the horse was originally considered to be the only mammal, besides man, that suffered from spontaneous pulmonary emphysema as a result of chronic obstructive pulmonary disease (Gillespie and Tyler, 1969; Gerber, 1973). Chronic obstructive pulmonary disease (COPD) in horses is a cause of great economic loss being the most common pulmonary disease of the adult horse in temperate climates (Freeman *et al* 1993; Dixon *et al* 1995a). The aetiology/pathophysiology of pulmonary emphysematous changes found in advanced COPD was assumed to be excessive and/or unregulated

ENE activity (Dubin *et al* 1994) as occurs in human pulmonary emphysema (Laurell and Eriksson, 1963; Gadek *et al* 1981a).

1.5 The Importance of Proteolytic Regulation

The regulation of proteolytic enzymes in tissues by endogenous inhibitors is a critical requirement for maintenance of homeostasis without which, extensive host tissue damage would occur. These inhibitors are proteins and glycoproteins found primarily in the blood plasma (representing over 10% of total plasma proteins) although increasing numbers of intracellular inhibitors are being identified. Several classes of proteinase inhibitors exist to regulate the activity of the different classes of proteolytic enzymes present. One superfamily of inhibitors, the serpins (SERine Proteinase INhibitors), predominates with over 60 known members which are found in animals, plants and viruses (though not Prokaryotes) (Potempa *et al* 1994; Carrell and Stein, 1996).

1.6 Serine Proteinase Inhibitors

It is estimated that serpins have been undergoing divergent evolution for some 200 million years from a common ancestral inhibitor (Carrell *et al* 1986; Goodwin *et al* 1996). Their common features are a conserved tertiary structure containing a stressed loop present in an exposed position near the C-terminus and a free N-terminal segment both of which are available for peptide cleavage (Carrell *et al* 1986). Although they are mainly involved with proteinase inhibition, they have also been found to have many diverse functions such as hormone transportation, blood pressure

regulation, tumour suppression, cell differentiation, protein folding, cell migration, prohormone conversion and regulation of the complement cascade (reviewed in Potempa *et al* 1994).

1.6.1 General Serpin Structure

The extracellular serpin superfamily have molecular masses ranging from 45000 to 70000 and contain varying amounts of CHO and disulphide bonds. The CHO side chains appear to play little role in the specific activity of individual serpins but have a general function of increasing their plasma half life (West, 1986; Travis *et al* 1990; Guzdek *et al* 1990; Mast *et al* 1990). The critical structure of those serpins which have retained their proteolytic inhibitory activity is the presence of a mobile reactive site loop (RSL) (Travis *et al* 1990; Lawrence *et al* 1994) which acts as a bait region for the respective proteinase. Minor changes in the amino acid sequence of the RSL have profound changes on the binding specificities and/or inhibitory functions of these proteins. The alpha-1-proteinase inhibitor mutation $PiM_{pittsburgh}$ in which the P1 residue methionine-358 is replaced by an arginine residue results in the inhibitory specificity of the molecule changing from HNE to thrombin effecting a fatal bleeding disorder in humans (Owen *et al* 1983). The most studied member of the inhibitory serpins is alpha-1-proteinase inhibitor (API) whose main role appears to be the regulation of neutrophil elastase, especially in the lower respiratory tract (Gadek *et al* 1981a; Janoff, 1985a;).

1.7 Human Alpha-1-Proteinase Inhibitor

1.7.1 Historical Context

Alpha-1-proteinase inhibitor was discovered when it was realised that a hereditary disorder resulting in the development of premature emphysema was linked to a decrease in the α1-globulin band of protein on one dimensional agarose gel electrophoresis of human serum (Laurell and Eriksson, 1963). It had previously been shown that 90% of the α1-globulin band consisted of a single protein which could inhibit the enzymatic properties of trypsin, hence the protein was initially named alpha-1-antitrypsin (Jacobsson, 1955). It was not until pulmonary emphysema was shown to be induced by intratracheal instillation of proteolytic enzymes, especially purified HNE, that the true target enzyme of API was determined (Hayes *et al* 1975; Janoff, 1985b). This lead to the realisation that although alpha-1-antitrypsin does indeed inhibit trypsin, it is a misnomer in that the physiological target enzyme is neutrophil elastase. As a result the term alpha-1-proteinase inhibitor is now preferred.

1.7.2 Alpha-1-Proteinase Inhibitor Gene Expression

The human API gene is located on the q arm of chromosome 14 (Sefton *et al* 1990), is 12.2 kb in length and comprises of seven exons (Crystal, 1989). The protein coding region is within exons II-V and encodes for a 418 amino acid product including a 24 amino acid signal peptide from exon II (Long *et al* 1984) which enables endoplasmic transport and processing in the Golgi apparatus prior to secretion into the plasma (Brantly *et al* 1988). The encoding for the P_1 methionine-358 residue on the reactive site loop of API is within exon V (Loebermann *et al* 1984). Two of the three

glycosylation sites are encoded in exon II, the other in exon III. The API gene is expressed in a variety of cells; peripheral blood monocytes, pulmonary and breast macrophages (Perlmutter *et al* 1985), neutrophils (Paakko *et al* 1996), alveolar epithelial cells (Venembre *et al* 1994; Cichy *et al* 1997), intestinal epithelium (Molmenti *et al* 1993) and cornea (Twining *et al* 1994), but most abundantly in hepatocytes (Alper *et al* 1980) which contain 200 times more mRNA than other cells (Rogers *et al* 1983). Transcription of the API gene results in three major mRNA products, 1.6, 1.8 and 2.0kb in length. The shortest, 1.6kb, mRNA is exclusively found in hepatocytes and enterocytes with the transcription start site located within exon IC (Perlino *et al* 1987). The two longer mRNA products, 1.8 and 2.0kb, found in mononuclear phagocytes and other non-hepatic, non-enterocytic API producing cells, are in exons IA and IB respectively, providing two alternative transcription start sites (Hafeez *et al* 1992). The protein product from all these mRNA species is however identical. These variable transcription start sites may explain why different API producing cells respond differently in terms of API synthesis to identical stimuli (Koj *et al* 1991; Sallenave *et al* 1997b).

Over 2g of API are secreted daily into human plasma (Jeppsson *et al* 1978; Jones *et al* 1978) to maintain a basal concentration of 1-1.3mg/ml in the normal adult, virtually all of this active circulating plasma API being derived from hepatic synthesis (Travis and Salvesen, 1983; Carrell, 1986). As API is one of the acute phase inflammatory proteins the plasma concentration can rise rapidly, up to four times the basal level in response to liberated inflammatory mediators (Dickson and Alper, 1974; Perlmutter,

1996). This rise is due to an increase in actual synthesis, mainly in the liver and not just release of preformed stored protein (Schulze *et al* 1990). As might be expected for an acute phase protein, production is upregulated by IL-6 in hepatocytes and blood monocytes (Perlmutter *et al* 1989; Hafeez *et al* 1992). Interestingly, during basal conditions, hepatocyte API transcription is from exon IC, but during upregulation by IL-6 transcription also occurs from exon IA as in non-hepatic, non-enterocytic cell types (Hafeez *et al* 1992). The IL-6 induced API upregulation in hepatocytes also appears resistant to corticosteroid inhibition (Castell *et al* 1988; Perlmutter *et al* 1989; Hafeez *et al* 1992). In monocytes API synthesis is upregulated by neutrophil elastase (NE) and lipopolysaccharide (LPS) in an additive manner (Perlmutter and Punsal, 1988). Neutrophil elastase upregulates synthesis and LPS by an as yet unknown mechanism (Perlmutter *et al* 1988) (Perlmutter and Punsal, 1988).

1.7.3 Structure

Alpha-1-proteinase inhibitor is a 52 kDa, single chain, 394 amino acid globular glycoprotein with three externally located asparagine linked CHO side chains (Carrell *et al* 1982; Loebermann *et al* 1984; Eriksson, 1996). The side chains, located at Asn-46, Asn-83 and Asn-247, are variably biantennary or triantennary complex carbohydrates and therefore result in microheterogeneity visible on gel electrophoresis (Carrell and Owen, 1979; Vaughan and Carrell, 1981; Mackiewicz *et al* 1993). The CHO side chains present in the native glycosylated form of API somehow prevent renal filtration, resulting in a plasma half life of 5 days compared to 2 days for unglycosylated recombinant API (Mast *et al* 1990). Incompletely glycosylated

variants of API are found to have identical second order association rates with HNE when compared to native API but are found to be more heat sensitive than the mature protein (Guzdek *et al* 1990). These findings suggest that the CHO side chains are of little importance to the proteinase inhibitory function of API but play key roles in the general stabilisation of the protein molecule and the duration of its plasma half life.

The single amino acid chain is formed into nine α-helices (A-I) and three β-pleated sheets (A-C) which are arranged in such a way as to hold the reactive centre loop (the forth strand of β-sheet A) out from the rest of the molecule. The exposed reactive site loop acts as a 'bait region' (Carrell *et al* 1986) mimicking a peptide sequence susceptible to attack by the active site of serine proteinases, especially HNE. The 15 amino acid sequence of the RSL is critical to the specificity, rate of association and inhibition of target proteinases (Olson *et al* 1995; Djie *et al* 1996; Elliott *et al* 1996a). Full primary, secondary and tertiary structures of human API have been elucidated and the P_1, P_1' residues, which are located on the RSL, are methione-358 and serine-359 respectively. These two residues are held such that their peptide bond is greatly distorted resulting in the native inhibitor molecule being in a 'stressed' configuration (Figure 1.1) (Loebermann *et al* 1984).

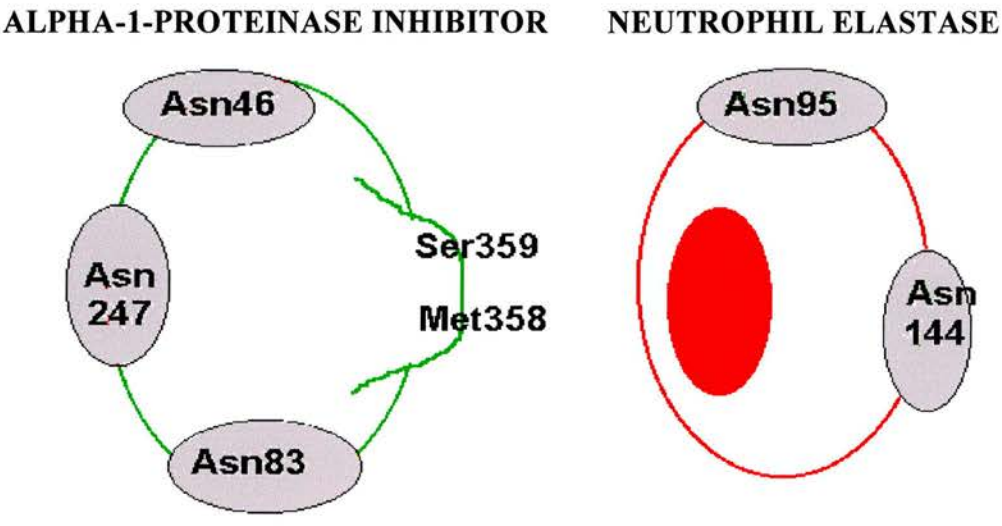

Figure 1.1 - Diagrammatic representation of alpha-1-proteinase inhibitor and neutrophil elastase. Grey ellipses represent asparaginyl (Asn) linked CHO side chains (numbers equal position of Asn on protein chain). Met358 and Ser359 are the P_1 and P_1' residues respectively situated on the reactive site loop of API. The red ellipse depicts the surface depression where the active site of NE is situated.

A critical feature of the serpins that separates them from families of smaller molecular weight inhibitors is the conformational polymorphism of the RSL, which, along with at least two β-pleated sheets, change conformation during the reaction with proteinases. It has been suggested that this property is responsible for the unique inhibitory mechanism of serpins and also the ability to form dimers and polymers (Wright, 1996).

1.7.4 Mechanism of Inhibition

The mobile RSL of API mimics the substrates of HNE so that the two molecules are strongly attracted to each other ($k_{ass} = 10^7\ M^{-1}\ s^{-1}$) by spatial conformity, hydrogen

bonds, electrostatic and Van de Waals forces (Branden and Tooze, 1991; Elliott *et al* 1996a). This is followed by the γ-OH group of HNE making a neucleophilic attack on the scissile P_1 Met-358, P_1' Ser-359 peptide bond of the RSL as would occur in a normal serine proteinase:peptide interaction. What happens next is not completely understood but it is thought that the two molecules form a tetrahedral intermediate which then follows one of two possible paths. The proteinase may, in some cases, cleave the inhibitor and dissociate from it resulting in active proteinase and inactive inhibitor, or, as occurs in the majority of cases, form an SDS stable enzyme:inhibitor complex possibly involving formation of a covalent bond via an acyl-enzyme structure (Wright, 1996).

Cleavage of the RSL produces a great conformational change in API such that the two once adjacent amino acid residues, P_1 and P_1', are moved to the opposite poles of the molecule 69Å apart, however, it is very controversial as to whether this occurs in stable proteinase:inhibitor complexes (Mast *et al* 1991b). Uncomplexed API in which proteolytic cleavage of the RSL has taken place results in increased heat stability of the molecule suggesting the active inhibitory form is in an energetically stressed state (Carrell *et al* 1986).

When HNE complexes with API, it does so in a pseudo-irreversible manner (Elliott *et al* 1996a) in a 1:1 molar ratio (Travis and Salvesen, 1983). Both molecules are inhibited when complexed and if dissociation does occur the resultant free API is usually in the relaxed state due to cleavage of the RSL at the P_1-P_1' bond and thus

unable to inhibit further HNE. For this reason API is known as a 'suicidal' inhibitor as each molecule can only inhibit once (Wright, 1996). Alpha-1-proteinase inhibitor does interact with other serine proteinases but with much slower association rates suggesting that regulation of HNE activity is its primary inhibitory role (Table 1.2) (Carrell *et al* 1986).

Serine proteinase	Association rate constant (k_{ass}) $M^{-1}s^{-1}$
Human neutrophil elastase	7×10^7
Porcine pancreatic elastase	1×10^5
Porcine pancreatic trypsin	4×10^4
Human thrombin	5×10^1
Human kallikrein	7×10^1
Human Factor Xa	2×10^2
Human Factor XIa	7×10^1
Human Factor XIIf	Nil
Human plasmin	2×10^2
Human cathepsin G	4×10^5

Table 1.2 - Variation in association rate of API with different serine proteinases (from Carrell and Boswell, 1986).

Any resultant API:serine proteinase complex is recognised by the serpin-enzyme complex (SEC) receptor (Pizzo *et al* 1988) located on hepatocytes, Kupffer cells and activated monocytes and macrophages (Perlmutter *et al* 1990a) which internalise and degrade it (Perlmutter *et al* 1990b; Joslin *et al* 1991a). This receptor is not specific for API:HNE complexes but also binds to antichymotrypsin, C1 inhibitor, antithrombin III and heparin cofactor II when complexed with serine proteinases (Pizzo *et al* 1988; Joslin *et al* 1991b). In addition several tachykinins such as substance P, amyloid-β peptide and bombesin have been also found to bind to the SEC receptor (Joslin *et al* 1991b). The SEC receptor recognises a pentapeptide domain equivalent to amino acid

residues 370-374 on API (Joslin *et al* 1991a) which has been shown to be conserved in other serpins (Joslin *et al* 1992). Recent studies have also shown the involvement of a low density lipoprotein in serpin:enzyme recognition, cellular internalisation and degradation (Poller *et al* 1995; Kounnas *et al* 1996). Despite the controversy over the precise mechanism of recognition it has been clearly demonstrated that serpin:enzyme complexes are rapidly removed from the circulation with a half life of about 12 minutes (Pizzo *et al* 1988) compared to 5 days for native API (Jones *et al* 1978; Mast *et al* 1990). Proteolytically inactivated inhibitors are also removed but less rapidly than the complex (Mast *et al* 1991b).

The API:HNE complex has been shown to be biologically active in that it is a potent chemoattractant for neutrophils (Banda *et al* 1988) and an upregulator of the API gene in monocytes and macrophages suggesting a role in inflammatory signalling and neutrophil activation (Perlmutter *et al* 1990a).

1.7.5 Oxidative and Proteolytic Inactivation of API

The RSL of API, by virtue of its amino acid structure and physical accessibility, is susceptible to both proteolytic and oxidative inactivation (Johnson and Travis, 1979; Mast *et al* 1991a). The P_1 Met-358 residue can be oxidised to methionine sulphoxide by a host of different oxidants found in macrophages (Mast *et al* 1991a), neutrophils (Ras *et al* 1992) and cigarette smoke (Travis *et al* 1980). Proteolytic cleavage of the RSL can be brought about by matrix metalloproteinases (Mast *et al* 1991a), neutrophil metalloproteinases (Ottonello *et al* 1994) and bacterial proteinases (Suter

and Chevallier, 1991; Potempa *et al* 1991a). These mechanisms greatly reduce the efficacy of API as an inhibitor and hence as a regulator of proteinases (Table 1.3) (Matheson *et al* 1982). These two mechanisms may therefore be important in the development of pulmonary emphysema in API sufficient individuals.

Enzyme	Native API	Oxidised API
Human neutrophil elastase	6.5×10^7	3.1×10^4
Human chymotrypsin	5.5×10^6	1.0×10^6
Porcine pancreatic elastase	1.0×10^5	Zero
Human trypsin	1.1×10^4	3.0×10^3
Human plasmin	1.9×10^2	Zero

Table 1.3 - Association rate constants (k_{ass} $M^{-1}s^{-1}$) of native and oxidised API with serine proteinases (from Matheson et. al. 1982).

1.7.6 Deficiency and Disease

In man several normal genetic variants, which all have circulating plasma levels of fully functional API within the standard range, are recognised and denoted as M due to their middle position of migration on isoelectric focusing gels (Allen *et al* 1974). However, there are at least 20 known API alleles associated with API plasma deficiency and several different mechanisms by which these mutants manifest themselves (Brantly *et al* 1988; Crystal *et al* 1989). The Z mutation, which was the first to be discovered (Laurell and Eriksson, 1963), is responsible for more than 95% of all the diagnosed cases of API deficiency (Brantly *et al* 1988). It is an autosomal recessive genetic defect found in the descendants of Northern Europeans and probably occurred in a single individual about 6000 years ago (Cox *et al* 1985). The prevalence of this mutant gene is relatively high suggesting that at least those with the

heterozygous genotype, if not the homozygous recessive as well, must have had some positive selection advantage over the normal genotype (Kueppers, 1972). Estimates put the prevalence of the MZ phenotype to be around 5% in Scandinavians, 1.5% in Britons, 1-2% of Southern Europeans and about 2.2% in the heterogeneous white population of the United States of America (Carrell and Owen, 1979).

The Z mutation is caused by a single base substitution ($Glu^{342} \rightarrow Lys$) resulting in the loss of a conserved negatively charged glutamic acid residue for a positively charged lysine which is thought to disrupt an ionic bond implicated in the stabilisation of the molecule (Loebermann *et al* 1984; Massi, 1996). There appears to be normal production of the protein in the hepatocyte and about 15% is successfully secreted into the plasma. The remaining 85% of the API becomes blocked in the rough endoplasmic reticulum of the hepatocytes prior to the final processing of the carbohydrate sidechains and so probably does not enter the Golgi complex (Hercz *et al* 1978; Hercz and Harpaz, 1980). This accumulation of protein in the hepatocytes is clearly visible on histological sections as perioic-acid-Schiff positive, diastase-resistant globules (Clausen *et al* 1984). Along with the reduced plasma concentration of API in the ZZ phenotype (Brantly *et al* 1991) there is also reduced ability to interact with and inhibit HNE (Ogushi *et al* 1987; Padrines *et al* 1989). The delay time of inhibition is the time required for almost complete inhibition of a proteinase *in vivo* and is a measure of whether an inhibitor will play an efficient proteolysis preventing function, values of less than 1 second are required to prevent uncontrolled HNE activity (Crystal, 1996). This value can be calculated for different phenotypes of API and also

different states of the molecule i.e. native or oxidised. The ZZ phenotype has a greater delay time of inhibition (Table 1.4) (Padrines *et al* 1989; Brantly *et al* 1991) which probably results in a reduced anti-elastase screen and a greater chance of NE induced pathology.

API Phenotype	Serum Concentration (μM)	PELF[a] Concentration (μM)	Association constant k_{ass} ($M^{-1}s^{-1}$)	Delay time of inhibition (sec)
M1M1	20-53	4.6	9.6×10^6	0.12
ZZ	3.4-7.0	0.46	4.6×10^6	2.40
SS	20-48	1.2	7.1×10^6	0.60
Oxidised M1M1	20-53	4.6	7.0×10^3	155.00

Table 1.4 - Comparison of concentrations and the kinetics of inhibition in different API phenotypes. [a] pulmonary epithelial lining fluid.

The second most common deficiency phenotype, the homozygous S allele is also a single base mutation $Glu^{264} \rightarrow Val$, again this disrupts a stabilising ionic bond within the molecule (Elliott *et al* 1996b). The loss of stability is even greater here and probably contributes to most of the nascent protein being degraded with no intracytoplasmic inclusions or pathology seen on histological examination of the liver (Carrell and Owen, 1979; Loebermann *et al* 1984). The SS phenotype has a plasma API concentration of 20-48μM (Brantly *et al* 1991). The critical level of plasma API concentration below which the lungs appear to be susceptible to excessive NE activity is about 11μM (Wewers *et al* 1987; Brantly *et al* 1991). If either the Z or S allele are combined with an M allele their respective plasma API levels are 15-42μM and 18-52μM and so pulmonary diseases are less likely. However, in the SZ phenotype the plasma levels vary from 10-23μM and about 10% of these individuals develop

premature emphysema (Brantly *et al* 1991). In both forms, ZZ and SZ, clinical signs of premature emphysema typically begin in the third to forth decade of life (Brantly *et al* 1988; Cox, 1989) the average age of onset being about 10 years sooner if the subject is a cigarette smoker. The latter case is probably due to oxidative inactivation of API directly by inhaled oxidants and indirectly from the oxidative radicals liberated from activated neutrophils which are attracted by chemotaxins released by alveolar macrophages in response to cigarette smoke (Carrell and Owen, 1979; Janus *et al* 1985; Gadek and Pacht, 1996a).

The ZZ phenotype results in clinically apparent liver disease in about 10% of juveniles (cholestatic hepatitis) and is also diagnosed in a number adults. The mutation induced structural instability in the ZZ phenotype results in the noncovalent insertion of the reactive site loop of one molecule into the A sheet of another, producing a dimer. This is followed by a cascade of events leading ultimately to polymerisation of ZZ API in the rough endoplasmic reticulum and precipitation (Lomas, 1994). These precipitates are seen as globular inclusions under light microscopy and are responsible for the 85% of ZZ API that is synthesised but not secreted. It is thought that even though these globules are unlikely to be directly toxic their presence disrupts the normal functions of hepatocytes and renders them more prone to external insults. As only a small number of infants are clinically affected investigators have looked for some predisposing factor or triggering device. It is now thought that the hepatitis C virus and other as yet unidentified viruses are responsible for this (reviewed in Massi, 1996).

Alpha-1-proteinase inhibitor deficiency has also been associated with a rare form of relapsing panniculitis, its role in the pathogenesis still remains obscure but as the initial pathological change is splaying of collagen bundles by neutrophils the link with potential elastase activity is apparent (Oriordan *et al* 1997).

1.8 Equine Alpha-1-Proteinase Inhibitor

1.8.1 Historical Context

The initial interest in equine API stemmed from the potential role of the horse as a disease model as it was considered the only animal besides man to suffer from spontaneous pulmonary emphysema (Gillespie and Tyler, 1969; Gerber, 1973). The interest was heightened when it was found that horses mainly developed pulmonary emphysema in neutrophil associated lung diseases such as chronic obstructive pulmonary disease (COPD) (Kaup *et al* 1990; Robinson *et al* 1996). However, to date, no deficiency of API has been reported in the horse so there is no true analogy with human API deficiency.

1.8.2 Genetics, Structure and Function

Unlike human API which is transcribed from a single gene producing one plasma glycoprotein (Crystal, 1989), equine API consists of four or five plasma glycoproteins controlled by four closely linked loci named serine proteinase inhibitor (Spi) 1, 2, 3 and 4. Limited sequence data suggests that these four loci are all derived from the identical ancestral gene that gave rise to human API (Patterson and Bell, 1989; Patterson *et al* 1991). Peptide and immunopeptide mapping confirms the close

relationship between the four loci but also reveals that Spi1 and 2 are more closely related to each other as are Spi3 and 4 indicating that these two pairs probably diverged from two closely related genes (Patterson *et al* 1991). To date 22 equine API haplotypes have been isolated, two of these being confined purely to *Equus przewalskii* (Putt and Whitehouse, 1983; Patterson *et al* 1991). Of the remaining 20 found in *E. callabus* eight are common in the Thoroughbred; F, G, I, L, N, S, T (also known as S_2) and U (Pollitt and Bell, 1983a; Pollitt and Bell, 1983b). The Spi4 gene is only expressed in 5 of the 22 haplotypes; F, J, T, V and Z (Patterson *et al* 1991). The Pi system is considered to be the most complex protein system in the horse and its inheritance has been used for study of the phylogenetic relationships of the equidae (Putt and Whitehouse, 1983) and for parentage verification (Bell *et al* 1984).

Equine APIs are glycoproteins with asparagine linked bi-antennary CHO side chains present on all of the Spi proteins (Patterson and Bell, 1990). The Spi 3 protein consists of two isoforms, A and B, which differ in molecular weight and acidity but have identical N-terminal amino acid sequences (Patterson *et al* 1991). The difference in molecular weight and acidity partially correlates with that of two extra terminal sialic acid residues on a CHO side chains of the most acidic Spi3 (Patterson and Bell, 1986; Patterson and Bell, 1990). The N-terminal amino acid sequences of the Spi proteins were initially investigated by two groups, one suggested that they are all identical over the first 32 residues (Potempa *et al* 1991b) while the other found significant differences in each Spi protein examined (Patterson *et al* 1991). A more recent study in this laboratory agrees with the latter (Pemberton *et al* 1993).

All of the Spi proteins inhibit ENE 2A, 2B and other proteinases but have differing rates of association and inhibition (Potempa *et al* 1991b) (Pemberton, A.D., personal communication). One interesting species difference between human and equine API is that in the horse complexes of API and ENE do not stimulate chemotaxis of equine neutrophils suggesting that the complex is not involved in the migration of equine neutrophils to sites of inflammation (Scudamore *et al* 1993). Biochemical and other differences may be due to variations in their respective structures (Table 1.5).

	LOCUS			
Criterion	**Spi1**	**Spi2**	**Spi3**	**Spi4**
Isoelectric point	4.02-4.43	4.18-4.35	3.73-4.08 (A) 3.92-4.26 (B)	4.12-4.27
Relative mass M_r	58000	61000	66000(A) 62200(B)	59700
Inhibitory activity	Trypsin/ chymotrypsin	Chymotrypsin	Trypsin	Trypsin
Oxidation sensitivity	Yes	No	No	No
P_1 residue	Methionine	Isoleucine	Alanine	Arginine
Terminal sialic acid content	4/5	4/5	8(A)/6(B)	4/5
CHO side chain structure	Biantennary	Biantennary	Bi- and Triantennary	Biantennary
Average plasma concentration:	1.4g/l	0.4g/l	1.6g/l	0.8g/l
Association rate constant with :				
ENE 2A ($M^{-1}s^{-1}$)	2.81×10^7	1.69×10^7	2.25×10^5	ND
ENE 2B ($M^{-1}s^{-1}$)	5.0×10^7	ND	5.2×10^5	ND

Table 1.5 - Summary of biochemical data for the Spi proteins of equine API.
ND = not determined. Pooled data from (Patterson *et al* 1991) (Potempa *et al* 1991b) and (Pemberton, A.D., unpublished data)

Spi1 with a P_1 of methionine, an average concentration of 1.4g/l, inhibitory activities against trypsin and chymotrypsin plus an association rate constant of 10^7 with ENE

2A and 2B is the functional equivalent of human API. Oxidation of the P_1 methionine to methionine sulphoxide reduces the association rate constant with ENE dramatically (from 10^7 to 0) (Potempa *et al* 1991b) again analogous to the human situation (Table 1.7). Biochemical, functional and structural studies of Spi1 from three different haplotypes, I, L and U, concluded that even though haplotype U has a larger molecular weight (M_r 57800) than I and L (M_r 55600) all had identical N-terminal amino acid sequences and association rate constants with ENE 2A (Pemberton *et al* 1993). The plasma concentration of Spi1 is the most consistent between haplotypes as determined by video densitometry, with Spi2 also having comparable plasma concentrations. However, the concentration of Spi3 varies between haplotypes by up to 50% (Patterson *et al* 1991). Both isoforms of Spi3 have been shown to be oxidation resistant (Patterson and Bell, 1989) due to the P_1 residue being alanine, not methionine (Potempa *et al* 1991b). This suggests that Spi2 and Spi4 would also be oxidation resistant as neither of them possess an oxidation sensitive P_1 residue (isoleucine and argenine respectively, Table 1.5).

From the biochemical and limited N-terminal amino acid data available and due to the conserved nature of the serpins, it is considered that analogies on the general structure of equine API can be derived from the more extensively investigated human API.

1.8.3 Equine Alpha-1-Proteinase Inhibitor and Disease

No deficiency of equine API has ever been recorded despite over 5500 samples being examined by one group alone (Pollitt and Bell, 1983b) and this is consistent with

experience in our own laboratory. Investigations into disease associations with equine API have fallen into two main categories, reproductive disorders such as endo- and pyometritis and pulmonary disease, especially COPD. The common factor linking these diseases is the prominent role of the neutrophil in the inflammatory process (Haslett *et al* 1989; Bokoch, 1995; Downey *et al* 1995).

Endo- and pyometritis is characterised by intense neutrophilic infiltration of the endometrium (Watson, 1988). Studies in Thoroughbred mares with pyometritis have shown the frequency of the N haplotype, which is already high in the Thoroughbred population, was significantly increased compared to that of a large published population. Also, in mares with acute endometritis that persisted after treatment and sexual rest, the absence of both the S and T haplotypes was significant, suggesting that when present they may have a protective role (Pemberton *et al* 1994). The exact mechanism of this protected role has not yet been elucidated. Another study assessed the level of equine API in uterine flushings before and after experimentally induced endometritis in anoestrus, oestrus and dioestrus. Significant increases in both equine API and albumin compared to total protein were found in flushings of diseased mares during oestrus and dioestrus but not anoestrus. A positive correlation between the concentrations of API and albumin in the flushings led to the conclusion that the API present had diffused from the plasma rather than being locally synthesised (Scudamore *et al* 1994).

After lameness, respiratory disease is the commonest disorder resulting in wastage in the horse. In older animals (> 7 years) the most prevalent respiratory disease is COPD (Winder, 1987) which a Swiss study found histological evidence for in 37% of necropsied horses from a veterinary teaching hospital over a one year period (Winder and Von Fellenberg, 1987). Aetiologically COPD is considered to be the result of a late phase hypersensitivity response to inhaled antigens particularly the thermophilic moulds and actinomycetes that grow on poorly conserved hay and straw. As a result of this COPD is mainly found in temperate climates where horses are stabled for long periods, bedded on straw and fed hay (Robinson *et al* 1996). Diagnostically it is characterised by an increase in percentage and absolute number of neutrophils in broncho-alveolar lavage fluid (BALF) (Freeman *et al* 1993; Dixon *et al* 1995a). Horses with COPD can be induced into remission by environmental management, involving increasing ventilation and using bedding and feed that are free from sensitising antigens and allergens (Robinson *et al* 1996). During remission, the number of neutrophils found in BALF returns to that of unaffected horses (Dixon *et al* 1995b).

All studies of equine API with respect to lung disease have been confined to investigations of COPD. The concentrations of equine API and albumin in PELF from horses with active clinical signs of COPD were significantly higher than those of COPD positive horses in remission. The correlation between API and albumin in the PELF suggested that most of the API was derived from plasma (Milne *et al* 1994c) in agreement with studies on equine endometritis (Scudamore *et al* 1994). Further

studies uncovered a significantly lower proportion of Spi3B and a higher proportion of Spi1 in broncho alveolar lavage fluid (BALF) compared with serum in both control and COPD affected horses (Milne *et al* 1994a). This has been interpreted as a physiological phenomenon possibly due to proteolytic damage or preferential complex formation by Spi3. Equine APIs in BALF have also been shown to have a lower molecular weight than that of serum (1.5kDa) in control, symptomatic and asymptomatic COPD positive horses (Milne *et al* 1994b). This was initially proposed to be physiological as lack of evidence of bacterial contamination in the samples suggested that cleavage by bacterial proteinases was unlikely. However, more recent data suggests this may have been due to changes in the APIs induced by the method of concentration of the BALF prior to being subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). If the BALF concentration equipment used by Milne et al, 1994 (Microsep® concentrators, Filtron Technology Co., Northborough or Nanosep® concentrators, Intersep Filtration Systems, Wokingham, Berks., UK) which requires centrifugation is replaced by a method that does not require centrifugation (Nanosep™ N10 concentrators, Intersep Filtration Systems, Wokingham, Berks., UK) then the 1.5kDa molecular weight shift seen on 10% SDS-PAGE does not occur (Preliminary studies, M. P. Dagleish).

1.9 Comparison of Human and Equine Susceptibility to Emphysema

Ultrastructural studies in horses with COPD show focal changes in the alveolar region including necrosis of type I epithelial cells, variable alveolar fibrosis with type II

epithelial transformation and emphysema or hyperinflation with an increase in number of Kohn's pores. The alveolar changes are mostly in the peribronchiolar region and emphysema was common in areas with bronchiolar obstruction (Kaup *et al* 1990). In humans emphysema is mainly centrilobular in distribution with neutrophil associated pulmonary disorders or panacinar with genetic API deficiency (Snider, 1992; Tetley, 1993; Travis *et al* 1994). Pulmonary emphysema has been shown to be due to an imbalance in proteinase:antiproteinase activity in humans (Laurell and Eriksson, 1963), and experimentally in dogs (Janoff *et al* 1977; Janoff *et al* 1979), hamsters (Hayes *et al* 1975) and mice (Starcher and Williams, 1989). The different patterns in the horse and human may therefore be due to species differences in total synthesis of both neutrophil elastase and API or to different concentration or functional activities and interactions of the two molecules.

Horses appear to develop pulmonary emphysema in neutrophil associated disorders such as COPD with a severity of histological changes comparable to the degree and extent of clinical findings (Kaup *et al* 1990). However, even in the most severe cases of equine COPD the extent of tissue damage and pulmonary emphysema is much less than that found in human chronic obstructive airway diseases (Creagh and Krausz, 1992). This suggests that the equine lung is more protected from damage induced by the host neutrophils which might due to a more favourable anti-proteinase quota in the proteinase:anti-proteinase equilibrium. This could arise from two potential mechanisms 1) a lower level of elastase in equine neutrophils (Dubin and Koj, 1986) or reduced release of mediator resulting in a reduced amount of local proteolysis in an

inflammatory situation, or 2) increased local equine API producing a more efficient anti-elastase screen in the lower respiratory tract of horses, or a combination of the two.

It is assumed that plasma equine API is mainly derived from hepatic synthesis as has been shown in humans (Alper *et al* 1980; Hafeez *et al* 1992), there is no direct experimental evidence to substantiate this but API has been localised in equine liver tissue by immunohistochemical means (Winder *et al* 1989). However, it has been shown that API present in the PELF of horses is probably derived from plasma API (Milne *et al* 1994c). This suggests that a greater concentration of plasma API would result in a greater concentration of API in PELF hence a more effective anti-elastase screen. The discovery of the synthesis and release of API from many extrahepatic human tissues suggests that the horse may also have API synthesising extrahepatic cells which could contribute significant amounts of protein locally for example in the alveoli.

Equine neutrophils are believed to contain much less NE (Dubin *et al* 1976) than human neutrophils (Campbell *et al* 1989) and this has been proposed as a possible reason for different patterns of pulmonary emphysema in the two species. However, the amount of ENE in equine neutrophils was derived by evaluation of elastinolytic activity of neutrophil extracts, whereas the human figure was derived by immunological means independent of the activity of NE. The discovery of horse leukocyte elastase inhibitor (HLEI), an intracellular NE inhibitor within the cytosol of

equine neutrophils (Potempa *et al* 1988; Kordula *et al* 1993) along with evidence that human neutrophils have been shown to express the API gene and produce API (duBois *et al* 1991), brings the accepted figure of NE in an equine neutrophil in to question as some may be inhibited during the extraction process and so not be available for detection by means of its activity.

In addition equine neutrophils have been shown to differ in release and generation characteristics from human neutrophils in their response to identical secretagogues (Brazil *et al* 1998). If the kinetics of release of NE and synergistic products such as superoxide anions (SOA) (Ottonello *et al* 1992; Ras *et al* 1992) differ, in that significantly lower amounts are released or over a longer time scale, their effects might be more easily inhibited and this could contribute to an altered proteinase:antiproteinase balance.

1.10 Aims of this Study

The aims of this study are to evaluate aspects of the equine proteinase:antiproteinase equilibrium, in an attempt to better understand the reason for the differences in patterns of pulmonary emphysema between man and horse. Specific antibodies for equine API and equine neutrophil elastase were developed. These antibodies were used to:

i) evaluate equine tissues and peripheral blood leukocytes for the possibility of significant local production of extrahepatic API.

ii) re-examine the accepted theory that equine peripheral blood neutrophils are relatively deficient in NE compared to human neutrophils by a method independent of NE activity.

iii) examine the kinetics of release of ENE 2A from Percoll purified equine peripheral blood neutrophils stimulated with a secretagogue and to relate this to concurrent superoxide anion generation.

Chapter 2

MATERIALS AND METHODS

2.1 Reagents and Solutions

All reagents were from Sigma Chemical Co. Ltd., Dorset, UK unless otherwise stated. Phosphate buffered saline (PBS) was prepared as described in Appendix 1.

2.2 Experimental Animals

2.2.1 Mice

Female balb/c mice were obtained from Bantam and Kingman Universal, Hull, UK, fed a standard pelleted diet and given water *ad libitum.* For monoclonal antibody production mice were deeply anaesthetised in an induction chamber with halothane (Rhone Merieux, Dublin, Ireland) prior to antigen inoculation. Mice were, similarly, deeply anaesthetised and killed by sectioning of the brachial artery and exsanguination and cervical dislocation, the spleen was then collected aseptically.

2.2.2 Sheep

One year old Suffolk cross sheep (from the Moredun Research Institute, Edinburgh, UK), were fed hay plus a standard sheep concentrate (16% protein) at 0.25Kg/ sheep per day and given water *ad libitum.* All pre-mortem blood sampling was by jugular venipuncture. Sheep were killed by jugular infusion of pentobarbitone sodium B.P. Vet (Rhone Merieux, Dublin, Ireland) followed by sectioning of the carotid artery and jugular vein to allow collection of blood for serum harvesting.

2.2.3 Horses

Horses were from the Royal (Dick) School of Veterinary Studies research herd, 2 geldings, 4 mares with ages ranging from 8 to 29 years, mean 15.8 years. All were housed in single stables, bedded on soft wood shavings, fed haylage or big bale silage (supplied by various local proprietors) and given water *ad libitum*. Blood sampling was by jugular venipuncture.

2.3 Sampling and Storage of Serum

Sera from all species was harvested by allowing blood to clot at 37°C for 30-60 minutes. The clot was then separated from the sides of the collection vessel, cut into several smaller fragments and stored at 4°C overnight to allow the clot to contract. Serum was harvested, aliquoted (mice 50µl, sheep and horses 1-50ml) and stored at -20°C until required. Equine serum, as a source of API, was obtained from horses presented for post-mortem examination, collection of blood was within 20 minutes of death after euthanasia by jugular infusion of Somulose (Arnolds Veterinary Products, Shrewsbury, UK) followed by sectioning of the carotid artery and jugular vein exsanguination. Equine plasma was collected into vessels containing 100ml sterile 1.36% ethylenediaminetetraacetic acid (EDTA) per litre of blood, the plasma being removed after free sedimentation of erythrocytes. Remaining erythrocytes were removed by centrifugation 1000 x g (Heraeus Megafuge 1.0R, Heraeus Equipment Ltd., Essex, UK), plasma was stored (1-50ml) at -20°C until required.

2.4 Determination of Purified Protein Concentration

2.4.1 Bicinchonic Protein Assay

The microtiter plate protocol recommended by the manufacturer (BCA Protein Assay Reagent, Pierce, Rockford, Illinois, US) was followed to determine protein concentrations. Absorbance of samples and standard curves was read at 570nm on a MR7000 ELISA plate reader (Dynex Technologies, Billingshurst, West Sussex, UK). The standard curve (bovine serum albumin (BSA) range of 15.6-2000μg/ml) and unknown samples were prepared in triplicate. Microsoft Excel 5.0a software was utilised to derive sample protein concentrations from the standard curve.

2.4.2 Ultra-Violet Spectrophotometry

Protein concentration was also determined by measuring the absorbance of a solution at wavelengths of 280nm and 260nm using a Beckman spectrophotometer DU 650 (Beckman Instruments, Inc., CA, US). The absorbance values were used in the following equation to determine protein concentration:

$$\text{Protein concentration} = (1.55 \times A_{280nm}) - (0.77 \times A_{260nm}) \text{ mg/ml.}$$

2.5 Electrophoresis

All gels were cast and run using the Bio-Rad Mini-Protean II system (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Gels and running conditions were based on modifications of previously published methods (Paakko *et al* 1990) (Krugliak *et al* 1986).

2.5.1 Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Buffers and chemicals:

1. Acrylamide- 30% acrylamide/ 0.8% bis-acrylamide
2. Resolving buffer- 1M Tris-HCl titrated to pH 8.8
3. Stacking buffer- 0.5M Tris-HCl titrated to pH 6.8
4. Sodium dodecylsulphate (SDS)- 10% w/v in dH_2O
5. Ammonium persulphate (APS)- 10% w/v in dH_2O (made fresh daily)
6. N,N,N',N'-Tetramethylethylenediamine (Temed)
7. Tank buffer- 0.3% Tris, 1.44% glycine, 0.1% SDS pH 8.3
8. Sample buffer (reducing)-

Stacking buffer	2.5ml
10% SDS	4.0ml
2-Mercaptoethanol	1.0ml
Glycerol	2.0ml
Bromophenol blue (0.1% w/v)	0.5ml

The solutions were mixed according to the table below to give gels with the required resolution.

Solution	Stacking Gel (4%)	Resolving Gel (10%)	Resolving Gel (12%)
Resolving buffer (ml)	-	2.5	2.5
Stacking buffer (ml)	1.4	-	-
Acrylamide (ml)	0.72	3.35	4.1
Water (ml)	2.95	3.05	2.3
SDS (ml)	0.28	1.0	1.0
Temed (µl)	10	10	10
APS (µl)	100	100	100

Table 2.1 - Composition of ingredients for different SDS gels for PAGE.

Samples in reducing buffer were heated to 100°C for 3 minutes before loading onto gels. Gels were run at 200V for approximately 45 minutes, electrophoresis was

stopped when the bromophenol blue dye front was within 2mm of the bottom of the gel.

2.5.2 Native-PAGE

Buffers and chemicals:

1. Acrylamide- 30% acrylamide/ 0.8% bis-acrylamide.

2. Gel buffer- 1.5M Tris (25ml)

2-Mercaptoethanol (75µl)

Temed (0.5ml)

dH_2O was added to a volume of 70ml then titrated to pH 7.8 with 20% H_2SO_4 and made up to 100ml total volume.

3. Ammonium persulphate- 0.2% w/v in dH_2O.

4. Blue dextran solution- 1% w/v in dH_2O.

5. Tank buffer- 65mM Tris-borate, pH 9.0.

7.87g Tris

1g Boric acid -made up to 1L with dH_2O

Solution	Stacking Gel	Resolving Gel (11.2%)
Acrylamide (ml)	0.5	4.48
Gel buffer (ml)	1.0	3.0
Ammonium persulphate (ml)	1.0	3.0
Blue dextran solution (ml)	1.5	-
dH_2O (ml)	-	1.52
Temed (µl)	2.0	-

Table 2.2 - Composition of ingredients for 11.2% native gel.

Non-reducing sample buffer was made by substituting dH_2O for 2-mercaptoethanol in the reducing buffer described above. Gels were run at 250V for approximately 50 minutes, electrophoresis was stopped when the bromophenol blue dye front was within 2mm of the bottom of the gel.

2.6 Visualisation of Electrophoresed Proteins

2.6.1 Coomassie Brilliant Blue

Fixation and staining of protein on polyacrylamide gels was with 0.25% w/v Coomassie brilliant blue (R-250) dissolved in 45% methanol/ 10% glacial acetic acid in dH_2O for 15 minutes. Gels were destained with 17% methanol/ 5% glacial acetic acid in dH_2O until all background pigment was removed allowing clear visualisation of protein bands.

2.6.2 Short Silver Stain

Gels were fixed with 50% methanol/ 10% glacial acetic acid in dH_2O for 15 minutes, washed in 5% methanol/ 7% glacial acetic acid in dH_2O for 10 minutes (twice), followed by two 5 minute washes in dH_2O. Dithiothreitol solution (0.5mg in 100ml dH_2O) was added for 15 minutes to reduce the proteins followed by three 5 minute washes in dH_2O. Gels were incubated in 0.1% silver nitrate in dH_2O for 20 minutes, rinsed in dH_2O for 30 seconds and developed in a solution of 50µl of 40% formaldehyde in 100ml of 3% Na_2CO_3. Band development was assessed by direct visualisation of the gel over a light box and inhibited by addition of citric acid (monohydrate) until the effervescence stopped.

2.6.3 Trypsin Inhibitor Gels

To identify the trypsin inhibitory properties of API and to identify Spi isoforms (1, 3A and 3B) samples were first separated by 11.2% native-PAGE. Gels were then incubated with chymotrypsin free bovine pancreatic trypsin (70μg/ml in 50mM phosphate buffer, pH 7.4) for 30 minutes at 37°C followed by exposure to a mixture of N-acetyl phenylalanine-β-naphthyl ester (APNE, 5mg in 2ml of dimethylformamide) and Fast Blue salt (10mg in 18ml of 50mM phosphate buffer, pH 7.4). On development, proteins with trypsin inhibitory properties are seen as clear bands against a red background as the trypsin is inhibited from cleaving APNE, one of the products of which reacts with fast blue to form the red colouration. Once staining is complete gels were washed 3-4 times in dH_2O then placed in 2% acetic acid to inhibit further development.

2.6.4 Substrate Gels to Assess Proteolysis

Proteins were separated by 10% SDS-PAGE under non-reducing conditions with 0.2% azocasein incorporated into the resolving gel (Horie *et al* 1984). Following electrophoresis gels were washed 3 times in 2.5% Triton-X 100 for 30 minutes before overnight incubation in PBS. The resultant gels were stained with Coomassie brilliant blue solution and destained as described above; proteolysis of azocasein in the gels being seen as clear bands against a blue background.

2.7 Western Blotting

Proteins were transferred to polyvinylenedifluoride membrane (Immobilon-P) after native-PAGE or SDS-PAGE using a semi-dry blotting technique and a triple buffer method (Kyhse-Anderson, 1984):

Anode Buffer 1-	pH 10.4, 0.3M Tris in 20% (v/v) methanol.
Anode Buffer 2-	pH 10.4, 25mM Tris in 20% (v/v) methanol.
Cathode Buffer-	pH 9.4, 25mM Tris, 40mM 6-amino-n-hexonic acid in 20% (v/v) methanol.

Chromatography paper (3mm thick, Whatman International Ltd., Kent, UK) was cut to generously accommodate the area of the gel. Two sheets were soaked in anode buffer 1 and placed directly onto the graphite anode. One sheet soaked in anode buffer 2 was then placed on top of the other two followed by the inert membrane. The gel was trimmed and placed carefully over the membrane followed by three sheets of chromatography paper soaked in cathode buffer with the cathode plate being placed on top. Protein transfer was complete in 1 hour using a current of 0.8mA/cm^2 of gel (80mA/minigel). The membrane was air dried and stored between filter paper at 4°C until required.

2.8 Preparation of Affinity Columns

CNBr-activated Sepharose® 4B was soaked and washed several times in 0.1M HCl being degassed between each wash. On final degassing and draining away of supernatant the gel was allowed to cake and then added to a solution of affinity purified sheep anti-equine API antibody (15mg, see Chapter 3.2.1), purified equine

API (3.5mg, see Chapter 3.2.1) or ENE 2A (1mg, see Chapter 5.2.1) in 0.1M sodium carbonate buffer/0.5M NaCl, pH 8.3. The mixture was agitated in a universal container overnight at room temperature. Sodium azide was added (to 0.05%), the gel packed under gravity into a C-10/10 column (Pharmacia, Uppsala, Sweden) and stored at 4°C until required.

2.9 Purification of Spi1 and 3 from Affinity Purified Equine API

Ion exchange chromatography separates biomolecules based on differences in charge characteristics (cationic/anionic), and is dependent on buffer pH and the isoelectric point of the biomolecules.

Buffer A: 100mM NaH_2PO_4 (most acidic buffer)

Buffer B: 100mM $Na_2H_2PO_4$ (most basic buffer)

Buffer C: 1M NaCl

Buffer D: deionised water

A sample of affinity purified API (see Chapter 3.2.1) was diluted 1:1 with buffer (15% A, 5% B, 80% D) and loaded on to the Mono Q (strong anion) column. The buffer composition was then altered over time as shown in Table 2.3 with a constant flow rate (1ml/minute).

Time (min)	A (%)	B (%)	C (%)	D (%)
0.0	15.0	5.0	0.0	80.0
4.0	15.0	5.0	0.0	80.0
44.0	15.0	5.0	40.0	40.0
45.0	15.0	5.0	80.0	0.0
47.0	15.0	5.0	80.0	0.0
48.0	15.0	5.0	0.0	80.0
55.0	15.0	5.0	0.0	80.0

Table 2.3 - Changes in buffer composition with time in Mono Q column.

Protein content of eluants was monitored by absorbance at a wavelength of 280nm with 0.5ml aliquots collected during protein elution. Several runs of each haplotype of API were performed and each fraction was subjected to 11.2% native PAGE to assess identity and purity of the products. Pure preparations of Spi1 and 3 for each haplotype were pooled and the protein concentration determined by the Bicinchonic Protein Assay method (see above) and stored at -70°C until required.

2.10 Deglycosylation of Purified Spi Proteins

40µl of 1.2% SDS was added to a 100µl sample of purified Spi protein and placed in a boiling water bath for 15 minutes. On cooling 200µl of deglycosylation buffer (Appendix 1) was added along with 35µl (7 IU) of peptide N-glycosidase F (PNGase F) and incubated at 37°C overnight.

2.11 Purification of Mouse IgG_1 Antibodies

The NaCl concentration of maximum tissue culture supernatant was adjusted to 3.3M by addition of NaCl (9.64g/50ml) followed by addition of 1.0M sodium borate (pH 8.9) to 1/10 the volume of the supernatant/NaCl mixture. The resultant solution was passed through a Protein A column (Pharmacia, Uppsala, Sweden), 50ml was loaded per run, followed by 10 column volumes of 3.0M NaCl, 50mM sodium borate (pH 8.9) and 10 column volumes of 3.0M NaCl, 10mM sodium borate respectively. The bound antibody was eluted with 100mM glycine (pH 3.0). Antibody solutions were immediately neutralised by addition of 1M Tris, pH 10. Antibody solutions from several runs were pooled, desalted on a Sephadex G25 column (Pharmacia, Uppsala,

Sweden) into PBS+0.05% sodium azide and concentrated using an ultrafiltration cell (Amicon Ltd., Gloucestershire, UK). After evaluation of protein concentration (bicinchonic protein assay, see above) the concentration was adjusted to 2mg/ml then aliquoted and stored at -20°C until required.

2.12 Conjugation of Antibodies

2.12.1 Horse Radish Peroxidase Conjugate

Five milligrams of horse radish peroxidase (HRPO) was resuspended in 1.5ml dH_2O and incubated for 30 minutes at room temperature. 0.25ml of 0.1M sodium periodate was then added to the HRPO followed sequentially by a 20 minute incubation, addition of 0.25ml of 0.5M ethylene glycol to quench sodium periodate and a further 20 minutes incubation. The resultant mixture was dialysed overnight at 4°C using a 15,000 molecular weight cut off (MWCO) membrane (Fisher Scientific, Leicestershire, UK) against 1L of 5mM sodium acetate, pH 4.5, changing the solution once. Concurrently, 5.0mg of the antibody to be conjugated was dialysed overnight at 4°C using a 15,000 MWCO against 1L of 50mM $NaHCO_3$, pH 8.0 changing the solution once. To the dialysed antibody solution 4.0ml of 0.4M $NaHCO_3$, pH 10.5 was added followed by addition of the activated dialysed HRPO. The mixture was then incubated for 2 hours at room temperature. Sodium borohydride (0.50ml, 0.1M) was added and the reaction mixture incubated for a further 6 hours at 4°C. To separate the conjugated antibody from the unreacted HRPO the mixture was concentrated to 2ml and applied to a Supadex 100 column (Pharmacia, Uppsala, Sweden) equilibrated with PBS. The protein fraction was identified by absorbance of

light at 280nm, collected and pooled. Protein concentration and protein to enzyme ratios (RZ) were determined by UV spectrophotometry.

$$RZ = OD\ 403nm / OD\ 280nm$$

All HRPO conjugated antibodies were diluted 1:1 with glycerol as a cryoprotectant and stored at -20°C until required.

2.12.2 Fluorescent Antibody Conjugates

Affinity purified sheep antibodies and normal sheep IgG were conjugated to fluorescein isothiocyanate (FITC) and Cyanine3 (Cy3) using the FluoroTag™ FITC Conjugation Kit and FluoroLink-Ab™ Cy3™ Labelling Kit (Amersham Life Science, Inc., Arlington Heights, IL, US) respectively. In both cases the manufacturers' instructions were followed. Briefly, for sheep anti-ENE 2A antibody conjugation to Cy^3, 1mg of antibody in 1ml of PBS was added to coupling buffer (1M sodium carbonate buffer, pH 9.3) and thoroughly mixed by vortexing. To this, Cy3 dye was added and thoroughly mixed by vortexing followed by a 30 minute incubation period during which the mixture was vortexed every 10 minutes. The labelled antibody was then desalted and separated from the free dye by MW exclusion chromatography on a gel filtration column (provided in the kit) equilibrated with PBS, 0.1% sodium azide. Normal sheep IgG was labelled in an identical manner for use as a negative control. Sheep anti-equine API antibody conjugation to FITC was performed by reconstitution of the latter in 0.1M carbonate-bicarbonate buffer, pH 9.0 to give a ratio of FITC:IgG molecules of 10:1 respectively. The FITC was added dropwise while stirring to 4mg of sheep anti-equine API antibody in the same buffer and incubated for 6 hours at

room temperature in a light proof container with constant gentle agitation. The FITC labelled antibody was separated from the unreacted FITC by MW exclusion chromatography on a Sephadex G-25M column equilibrated with PBS, 0.05% sodium azide. Normal sheep IgG was labelled in an identical manner for use as a negative control. Final protein concentrations and fluorochrome to protein ratios were derived using UV spectrophotometry and the following equations:

$$\text{FITC Molar F/P} = 2.77 \times A_{495} / A_{280} - (0.35 \times A_{495})$$

$$\text{Cy3 Molar F/P} = (1.13 \times A_{552}) / [A_{280} - (0.08 \times A_{552})]$$

A = absorbance at a given wavelength of light (nm).

All fluorochrome conjugated antibodies were stored in PBS + 0.05% sodium azide in light proof containers at 4°C until required.

2.13 Cell Culture

Unless stated otherwise all cell culture associated with monoclonal antibody production was in plastic tissue culture flasks with vented caps (Costar, Cambridge, MA, US). Cells were cultured at 37°C in an humidified atmosphere of 5% CO_2 in air.

2.14 Cell Counting and Viability of Purified Neutrophils

Cell were counted by an automated cell counter (System 9000, Serono Baker Diagnostics, Pennsylvania, US) or in an improved Neubauer haemocytometer (Fisher Scientific, Leicestershire, UK). Cell viability was assessed based on trypan blue exclusion (10µl cells : 10µl dye).

2.15 Cytospin Preparation and Staining

To determine the percentage of neutrophils in preparations of isolated cells, 80µl of purified cells in platelet poor plasma (PPP) were centrifuged onto a glass slide for 3 minutes at 300 rpm (Shandon Cytospin 3, Southern Instruments Ltd., Runcorn, UK). Slides were air dried, fixed in methanol for 3 minutes and stained with Diff-Quik™ (B. M. Brown Ltd., Reading, Berks, UK). Slides were then washed with dH_2O to remove excess stain and mounted in DPX (BDH, Poole, UK). The percentage of neutrophils in each preparation was determined by counting 500 cells from randomly chosen high power (x 1000) fields.

For immunocytochemical evaluation, cells suspended in equine serum free media were centrifuged as above onto 3-aminopropyltriethoxysilane (TESPA) coated slides and fixed in freshly prepared 4% paraformaldehyde in PBS for 45 minutes at 45°C, transferred to 70% ethanol and stored at 4°C until required.

2.18 Statistical Analysis

Statistical analysis of release of superoxide anion, equine neutrophil elastase 2A and equine alpha-1-proteinase inhibitor from purified peripheral blood neutrophils was performed using Minitab 11.12 or GraphPad Prism 2.01 statistical software.

Chapter 3

GENERATION AND CHARACTERISATION OF SPECIFIC MOUSE MONOCLONAL AND SHEEP POLYCLONAL ANTIBODIES TO EQUINE ALPHA-1-PROTEINASE INHIBITOR

3.1 Introduction

Equine API has previously been examined biochemically to investigate the possible susceptibility to endometritis and pyometritis of Thoroughbred mares in relation to their API haplotype (Pemberton *et al* 1994). Equine API has also been quantitatively evaluated in uterine flushings, by immunological means, from mares at various stages in their reproductive cycle, as well as during experimentally induced endometritis (Scudamore *et al* 1994). This study showed statistically significant differences in the amount of equine API in the different stages of the normal reproductive cycle but none in the diseased mares. Similarly, equine API has been quantified in the pulmonary epithelial lining fluid (PELF) from the lungs of horses suffering from active symptomatic COPD and those in remission induced by environmental control (Milne *et al* 1994c). In this case the animals suffering from the active disease had a statistically significant higher concentration of equine API in their PELF compared to those in remission. These quantitative studies into physiological and disease situations used a polyclonal sheep anti-equine API antibody which, although successful in detecting equine API in a sandwich ELISA and for affinity purification, was not consistently useful for immunoblotting or immunohistochemistry.

To enable further investigation of the role of equine API in the normal physiology of the horse and with respect to the proteinase:anti-proteinase theory of pulmonary emphysema, an effective means of recognition and measurement is required that is consistently specific and sensitive in complex biological samples where other constituents may also be present (e.g. tissue, serum, cell preparations etc.). Generation of specific antibodies to the protein under investigation is one way of achieving this as they are able to detect their protein antigen with a specificity that is readily demonstrable on Western blots. Antibodies can be employed in both qualitative (Western blots, immunohistochemistry) and quantitative (ELISA) evaluations of their antigen.

For this reason a new panel of mouse monoclonal and sheep polyclonal antibodies to equine API were raised and characterised in an attempt to obtain antibodies which would be useful for studies involving techniques such as Western blots, tissue immunolocalisation and ELISA.

3.2 Materials and Methods

3.2.1 Production and Evaluation of Sheep Polyclonal Antibody to Equine API

Protein Purification. Equine API was purified from horse serum by affinity

chromatography on a sheep anti-equine API antibody column (kindly donated by Dr. Alan Pemberton). The antibody used for purification (15mg) was coupled to CNBr-activated Sepharose® 4B (5g) as described in Chapter 2.8.

Pooled equine serum was diluted 1:1 with PBS+0.05% sodium azide and loaded onto the column in 2ml aliquots. After equilibration with PBS+0.05% sodium azide, bound equine API was eluted with 2ml of 0.1M citric acid/0.5M NaCl/0.05% sodium azide, pH 2.5 and immediately neutralised by addition of 1M Tris, pH 10. Several runs were pooled, desalted on a Sephadex G25 (Pharmacia, Uppsala, Sweden) column into PBS+0.05% sodium azide, concentrated using a stirred ultrafiltration cell containing a membrane with a 30000 MW cut off (Amicon Ltd., Gloucestershire, UK). The protein concentration was adjusted to 1mg/ml after assessment by the bicinchonic protein assay method (Chapter 2.4.1). Aliquots were stored at -20°C until required. Purity was assessed by 11.2% native-PAGE.

Immunisation of Sheep with Affinity Purified Equine API. Sheep antisera were raised by mixing equal volumes of affinity purified equine API (100µg total protein) and Freund's complete adjuvant into an emulsion by passing it 20-30 times through a 21G needle. After blood sampling to provide control serum, the protein:adjuvant emulsion was injected into four separate subcutaneous sites in the sheep. Two subsequent immunisations over a 6 month period were performed as above except that the antigen was emulsified in Freund's incomplete adjuvant. A final injection of 100µg of

equine API in saline was given by the intra-muscular route 3 days before slaughter, collection and storage of antiserum (as described in Chapters 2.2.2 and 2.3).

Antibody Purification. Sheep polyclonal antibodies were purified by affinity chromatography. Equine API (3.5mg) was coupled to 1g of CNBr-activated Sepharose® 4B (Pharmacia, Uppsala, Sweden) and packed to produce an equine API affinity column for use in chromatography as described in Chapter 2.8.

Sheep anti-equine API antiserum was diluted 1:1 with PBS+0.05% sodium azide to reduce viscosity and loaded on to the equine API affinity column. After equilibration with PBS+0.05% sodium azide, bound material was eluted with 2ml of 0.1M citric acid/0.5M NaCl/0.05% sodium azide, pH 2.5. Antibody solutions were immediately neutralised by addition of 1M Tris, pH 10 to a final pH of 7.0. Optimal loading of the column was assessed by performing the above procedure with increasing sample volumes, starting with 1ml and increasing by 0.5ml each run, until saturation of the column was observed from the elution. The sample volume immediately previous to this was then used on all subsequent runs. Antibody solutions were pooled from several runs and concentrated using an ultrafiltration cell (Amicon Ltd., Gloucestershire, UK) before being desalted. Desalting was achieved by equilibrating a Sephadex G25 column (Pharmacia, Uppsala, Sweden) into PBS+0.05% sodium azide. The resultant antibody solution, now in PBS+0.05% sodium azide, was diluted to 1mg/ml after protein concentration evaluation (by the bicinchonic protein assay,

Chapter 2.4.1) and stored either with or without glycerol (1:1 ratio) at -20°C until required.

3.2.2 Characterisation of the Sheep Anti-Equine API Antibody

The specificity of the antibody was evaluated by probing Western blots of purified equine API and native equine API in serum. Affinity purified whole equine API containing the same mixture of isoforms found in serum (1μg total protein in 5μl of native sample buffer) and equine serum (diluted 1:20 in native sample buffer, 5μl loaded) were subjected to native 11.2% PAGE with blue dextran incorporated into the stacking gel to retard albumin migration (Pemberton and John, 1993). Four identical gels were produced, one was stained with Coomassie Brilliant Blue R250, the second evaluated for trypsin inhibitory properties, while proteins from the others were transferred to a polyvinylidene difluoride membrane (Immobilon-P) by semi-dry Western blotting (Chapter 2.7) for further evaluation.

Probing of Western blots was achieved by initially blocking non-specific binding of the antibody with 3% dried skimmed milk (J Sainsbury, London, UK) in PBS (3%MPBS) for 60 minutes. The membrane was then incubated with the affinity purified sheep anti-equine API antibody, 2.5mg/ml in 3%MPBS (60 minutes) followed by washing 3 times in PBS+0.05% Tween 20 and once in PBS respectively for 5 minutes each. To localise sheep anti-equine API antibody, donkey anti-sheep IgG: horse radish peroxidase (HRPO) conjugate (Sigma Chemical Co., A-3415) was applied at a 1/7500 dilution in 3%MPBS for 60 minutes. Following an identical wash procedure as

before a 3,3'-diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate kit (Vector Laboratories, Peterborough, UK) was utilised as per manufacturer's instructions. On appearance of protein bands the membrane was washed in 3-4 changes of dH_2O, photographed and subsequently dried on filter paper prior to storage in a light proof container. An identical procedure was performed on the fourth Western blot except the primary antibody was substituted with 2.5μg/ml of normal sheep IgG to act as a negative control.

Sera from Thoroughbred (Tb) horses homozygous for the 8 common haplotypes of API; F, G, I, L, N, S, T and U (kindly provided by M. Scott, Animal Health Trust, Newmarket, UK) were diluted 1:20 in native buffer and 5μl of the resultant mixtures were electrophoresed, transferred to Immobilon-P and probed with the polyclonal sheep anti-equine API antibody as above.

3.2.3 Production and Evaluation of Mouse Monoclonal Antibodies to Equine API

Production of Mouse Monoclonal Antibodies to Equine API. Mice (BALB/c females) were immunised 4 times with affinity purified equine API (40μg total protein each immunisation) from the same stock as that used for immunising the sheep for polyclonal antibody production. The first immunisation was emulsified with Freund's complete adjuvant (total volume 0.2ml), the second and third with Freund's incomplete adjuvant, all being given by the subcutaneous route 4 weeks apart. Sera obtained from tail bleeds were screened for specific antibodies by enzyme linked immunosorbant assay (ELISA). Microtitre plates were coated with affinity purified

equine API (50µl/well of 1µg/ml solution in 0.1M carbonate/bicarbonate buffer, pH 9.6, Appendix 1) overnight at 4°C. Following six washes with PBS+0.05% $Tween_{20}$ (PBST), sera was diluted 1:100 with PBS and 50µl was added per well and incubated at room temperature (approx. 21°C) for 60 minutes. Wells were then washed 6 times with PBST as before. Detection of bound primary antibody was by addition of sheep anti-mouse IgG:HRPO conjugate (Sigma Chemical Co. A-5906) diluted 1:5000 in 4% normal sheep serum in PBST for 60 minutes followed after washing six times with PBST by the addition of 50µl per well of 3, 3', 5, 5'-tetramethylbenzidine (TMB, Dynex Technologies, Billingshurst, West Sussex, UK) substrate. Colour development was stopped by the addition of 25µl of 0.18M H_2SO_4 and the optical density (OD) measured at 490nm on a MR7000 ELISA plate reader (Dynex Technologies, Billingshurst, West Sussex, UK). Sera from mice taken before immunisation (pre-immune sera) was used as a negative control being substituted for test sera at the same dilution.

Mice with serum positive for anti-equine API antibodies on ELISA were given a fourth injection of soluble affinity purified equine API intraperitoneally 72 hours before harvesting of spleens and sera (Chapter 2.2.1). Splenic cells were separated by manipulation of splenic tissue between two sterile glass slides and placed in serum free tissue culture media (Appendix 2) and washed twice by centrifugation at 400 x g for 5 minutes. An identical wash procedure was performed on cells from a mouse myeloma cell line (NSO cells from BALB/c mice). All tissue culture solutions were prewarmed to 37°C prior to use. The two populations of cells were combined and 0.5g of

prewarmed PEG 4000 diluted 1:1 with serum free media was slowly added over 1 minute with continual stirring. A further 1ml of serum free media was added over the next 1 minute followed by 9.0ml over the next 2 minutes with continuous stirring. The cells were then centrifuged at 400 x g for 5 minutes (Harlow and Lane, 1988). The supernatant containing the PEG was removed and RPMI 1640 (Gibco BRL, Life Technologies Ltd., Paisley, UK) containing 20% foetal calf serum (FCS, Gibco BRL, Life Technologies Ltd., Paisley, UK) that had been previously screened to assess suitability for mouse myeloma cell growth (Appendix 2) was added. Following assessment of cell density (improved Neubauer haemocytometer, Fisher Scientific, Leicestershire, UK) dilutions of cells were made such that 100µl contained either 1 or 5 cells. Each 100µl sample was placed in the well of a 96 well plate and incubated in an humidified incubator at 37°C, 5% CO_2.

Supernatants from wells containing viable cells after hypoxanthine, aminopterin and thymidine selection were screened for specific antibodies using the ELISA method described above. Positive anti-equine API antibody producing cell lines, of which one (1.3.2) was produced by this study and another (1B) was kindly donated by Dr. Sybil M^{c}Aleese were cloned by limiting dilution.

The two cell lines were grown to produce maximum supernatant in static culture; the point where the maximum amount of antibody is found in the supernatant produced in a single tissue culture flask. Maximum supernatant was defined by a reduction in cell growth and viability as monitored by microscopic visualisation and decrease in pH as

indicated by yellowness of the tissue culture media (due to the presence of pH sensitive phenol red). Maximum supernatants were decanted and centrifuged at 10000 x g to remove cell debris and stored at -20°C until purification of antibodies.

Characterisation of Mouse Monoclonal Antibodies. A Mouse Monoclonal Antibody Isotyping Kit established that both monoclonal antibodies (1B and 1.3.2) belonged to the IgG_1 isotype. Initial characterisations of the two monoclonal antibodies were carried out using neat tissue culture supernatants as a source of anti-equine API antibody. Supernatants were used to probe Western blots made from a 1/20 dilution in native sample buffer of sera from the 8 common API haplotypes found in Thoroughbreds (kindly provided by M. Scott of the Animal Health Trust, Newmarket, UK) subjected to 11.2% native PAGE (described above and see Chapter 2.5.2).

Non-specific antibody binding was blocked with 0.5% $Tween_{80}$ in PBS (PBST80) for 60 minutes. Neat tissue culture supernatants from mAbs 1B and 1.3.2 were added to separate but identical Western blots for 90 minutes followed by three 5 minute washes in PBST80. Antibody binding was visualised by addition of goat anti-mouse IgG (Fab specific):HRPO conjugated antibody (Sigma Chemical Co., A 2304) diluted 1/5000 in PBST80 for 60 minutes followed by three 5 minute washes in PBST80 and incubated with DAB HRPO substrate (Vector Laboratories, Inc., Peterborough, UK). Once protein bands were visualised the membrane was washed in 3-4 changes of dH_2O, photographed and subsequently dried on filter paper before storage in a light proof container. For control purposes mAbs 1B and 1.3.2 were substituted with supernatant

from a mouse cell line producing IgG_1 raised to rat mast cell proteinase II (kindly provided by John Huntley, Moredun Research Institute, Edinburgh, UK).

3.2.4 Evaluation of Effects of Glycosylation on Recognition of Spi Isoforms by Monoclonal Antibodies

In order to further evaluate the specificities of the two monoclonal antibodies the Spi1 and 3 isoforms from haplotypes N, S and U were purified, deglycosylated, electrophoresed and transferred to Immobilon-P by Western blotting. The aim of this procedure was to compare the two monoclonal antibodies to assess if their difference in specificity was due to:

i) the antibodies binding to a CHO epitope rather than a peptide one.

ii) the more branching and therefore more bulky CHO side chains of Spi3 (Patterson and Bell, 1990) obstructing antibody binding to peptide epitopes close to them.

Purification of Isoforms Spi1 and 3 of Equine API. Equine API was purified from the sera of horses known to be homozygous for haplotypes N, S and U (kindly provided by M. Scott, Animal Health Trust, Newmarket, UK) by affinity chromatography (Chapters 3.2.1 and 2.8). Each haplotype of affinity purified API was then subjected to ion exchange chromatography using HPLC and a Mono Q column (Pharmacia, Uppsala, Sweden) as described in Chapter 2.9 to purify Spi1 and 3.

Deglycosylation and Western Blotting of Spi1 and 3. Previous studies have shown that equine API contains complex N-linked CHO side chains attached to asparagine

residues and these bonds are cleaved by peptide N-glycosylase F (PNGase) (Patterson and Bell, 1990). The purified Spi1 and 3 from haplotypes N, S and U were deglycosylated as described in Chapter 2.10.

Glycosylated and deglycosylated samples of identical haplotypes and isoforms (0.26μg/lane) were subjected to 10% SDS-PAGE under reducing conditions in adjacent lanes. Three identical gels were produced from the Spi1 and Spi3 isoforms. For each isoform one gel was stained by the short silver stain (Chapter 2.6.2), proteins from the other two were transferred to Immobilon-P by Western blotting (Chapter 2.7). One blot was probed with mAb 1B the other with mAb 1.3.2 as described above.

3.2.5 Purification of Anti-Equine API Monoclonal Antibody 1B

As mAb 1B recognised the greater number of Spi proteins and in a greater number of haplotypes it was considered that it would probably be the most useful monoclonal antibody against equine API for use in further investigations in tissues from untyped animals. For this reason and to be able to critically control the concentration of antibody employed in various procedures it was decided to purify mAb 1B. A Protein A hydrophobic interaction chromatography using a high salt, triple buffer technique was considered the best method for purification of a mouse IgG_1 antibody (Harlow and Lane, 1988) as described in Chapter 2.11.

A Western blot of the sera from the 8 common Tb haplotypes of API identical to that used to characterise the neat tissue culture supernatant (Chapter 3.2.3) was prepared

and probed in an identical manor except purified mAb 1B (40μg/ml in PBST80) replaced the neat tissue culture supernatant. A duplicate Western blot was probed by substituting 40μg/ml of normal mouse IgG_1 (MOPC 31c, Sigma Chemical Co., M 9035) for mAb 1B as a negative control.

3.3 Results

3.3.1 Production and Evaluation of Sheep Polyclonal Antibody to Equine API

The affinity purified equine API was not contaminated with other serum proteins when examined by Coomassie brilliant blue stained native 11.2% PAGE (Figure 3.1). A combination of Coomassie stained and trypsin inhibitor native PAGE allows individual identification of Spi1-3 (Milne *et al* 1994a) (Figure 3.2).

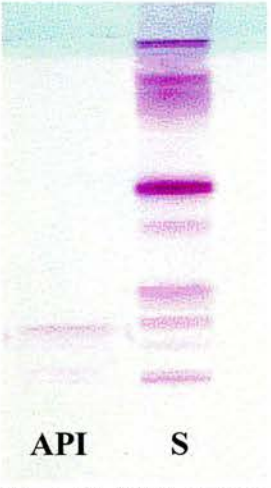

Figure 3.1 - Coomassie brilliant blue (R-250) stained 11.2% native PAGE of affinity purified equine API and equine serum (S).

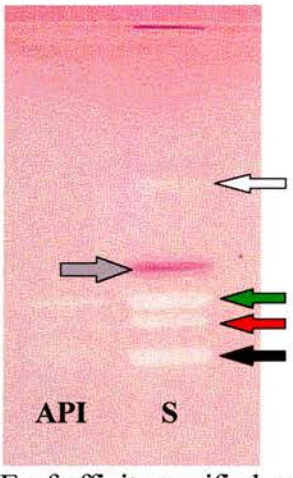

Figure 3.2 - 11.2% native PAGE of affinity purified equine API and equine serum (S) stained for trypsin inhibitory capacity. Spi1 (black arrow) migrates the furthest, Spi3A (green arrow) and Spi3B (red arrow) migrate the least, all are seen as clear bands on the pink background. Spi2 does not inhibit trypsin and so is not visible. The high MW band of inhibition corresponds to dimeric API (white arrow). Esterase is seen as a dark pink band (grey arrow).

Polyclonal sheep anti-equine API antibody specifically recognises Spi1, 2 and 3 in the presence of other serum proteins on a Western blot made from a gel identical to that in Figure 3.1 (Figure 3.3), dimeric API is also clearly recognised. Due to the presence of Spi4 in only 5 of the 22 known equine API haplotypes it was not considered further in these studies. The negative controls were unstained. The Western blot of the sera from the 8 common Tb haplotypes established that all Spi isoforms of equine API present were recognised by the polyclonal sheep anti-equine API, with only slight variation in intensity of staining between some isoforms (Figure 3.4a&b).

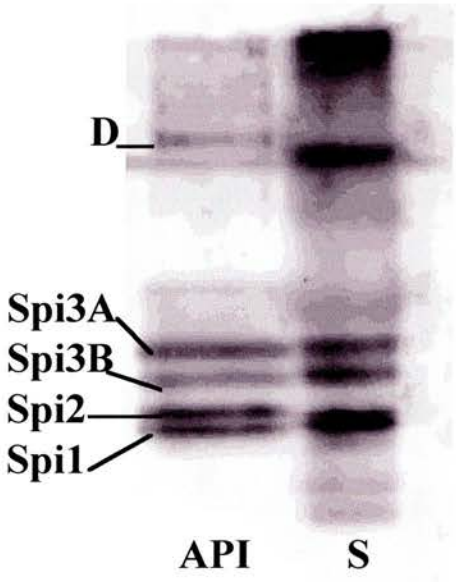

Figure 3.3 - Western blot made from an identical gel to that of Figure 3.1 and probed with sheep polyclonal anti-equine API antibody which specifically recognises all Spi proteins present and the dimeric form (D) in the presence of other serum proteins (S).

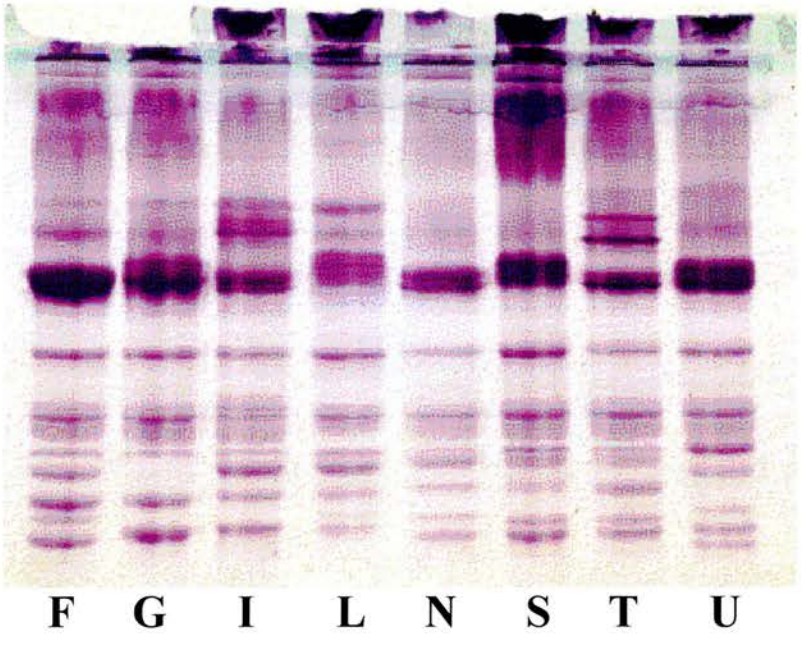

Figure 3.4a - Coomassie brilliant blue (R-250) stained 11.2% native-PAGE of the sera of the 8 common API haplotypes found in Thoroughbred horses.

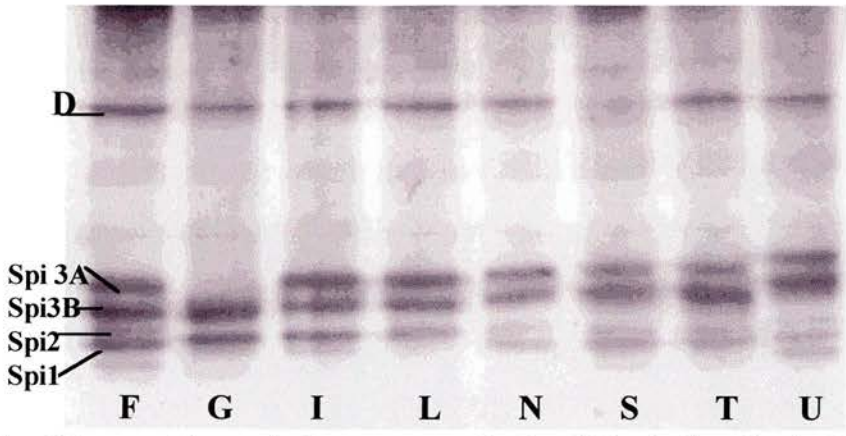

Figure 3.4b - Western blot made from an unstained gel identical to that in Figure 3.4a probed with sheep anti-equine API. All Spi isoforms were recognised including the dimeric form (D) with slight variations in the intensity of the positive staining, however 2-dimentional PAGE is needed to resolve some of these in certain haplotypes.

3.3.2 Evaluation of Mouse Monoclonal Antibodies to Equine API

Monoclonal antibody 1B (mAb 1B) specifically recognised all isoforms of API present in the sera of the 8 common Tb haplotypes but with variation in the intensity of immunostaining of individual Spi proteins of certain haplotypes (Figure 3.5). The Spi3 isoform of haplotypes N, S and T were less intensely immunostained by mAb 1B and dimeric API was recognised in all haplotypes except G. In contrast, mAb 1.3.2 only recognised Spi1 and 2 and then only in haplotypes G, I, L, S, T and U (Figure 3.6). Spi1 and 2 of haplotypes F and N and Spi3 of any haplotype were not recognised by mAb 1.3.2. The negative control preparation was devoid of any labelling.

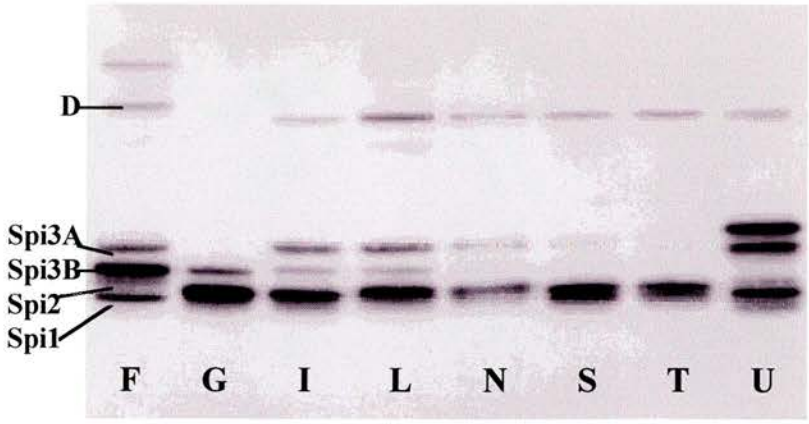

Figure 3.5 - Western blot from a gel identical to that in Figure 3.4a probed with mAb 1B. Note variation in intensity of staining of Spi isoforms in different haplotypes and lack of staining of dimer (D) in haplotype G.

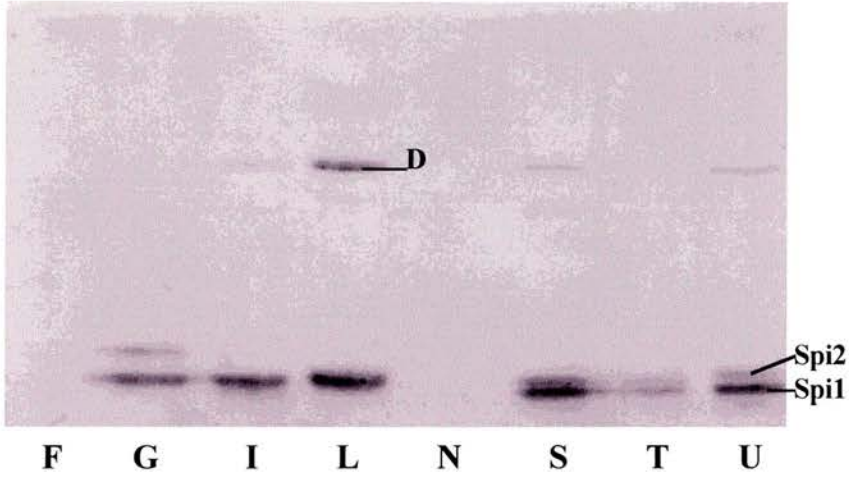

Figure 3.6 - Western blot from a gel identical to that in Figure 3.4a probed with mAb 1.3.2 which only recognises Spi1 and 2 and then not in all haplotypes examined. Recognition of API dimer (D) is also haplotype specific.

3.3.3 Evaluation of the Effect of Deglycosylation of Spi1 and 3 on the Specificity and Sensitivity of mAb 1B and 1.3.2

The isolated native glycosylated Spi1 isoforms (58kDa band) from all three haplotypes (N, S and U) showed greater than 95% purity with small amounts of contaminants of similar MWs (Figure 3.7). The two main products from deglycosylation of the Spi1 isoforms are seen as 49.5 and 46 kDa bands. Examination

of the Western blot probed with mAb 1B reveals that the antibody recognises all the glycosylated Spi1 isoforms from all three haplotypes and both of the deglycosylated products (Figure 3.8). However, the N haplotype still immunostains less intensely than those of S and U. This suggests that mAb 1B recognises a peptide epitope which is either less abundant or still, for some reason, less accessible to the antibody in the Spi1 isoform of haplotype N. When an identical Western blot was probed with mAb 1.3.2 both the glycosylated and deglycosylated Spi1 isoforms of haplotype N were just visible on immunostaining (Figure 3.9) compared to not at all when in serum (Figure 3.6), those of haplotypes S and T were clearly recognised by this antibody. These results suggest that mAb 1.3.2 also recognises a peptide epitope of Spi1 but this epitope is even less abundant and/or accessible in haplotype N than the epitope recognised by mAb 1B.

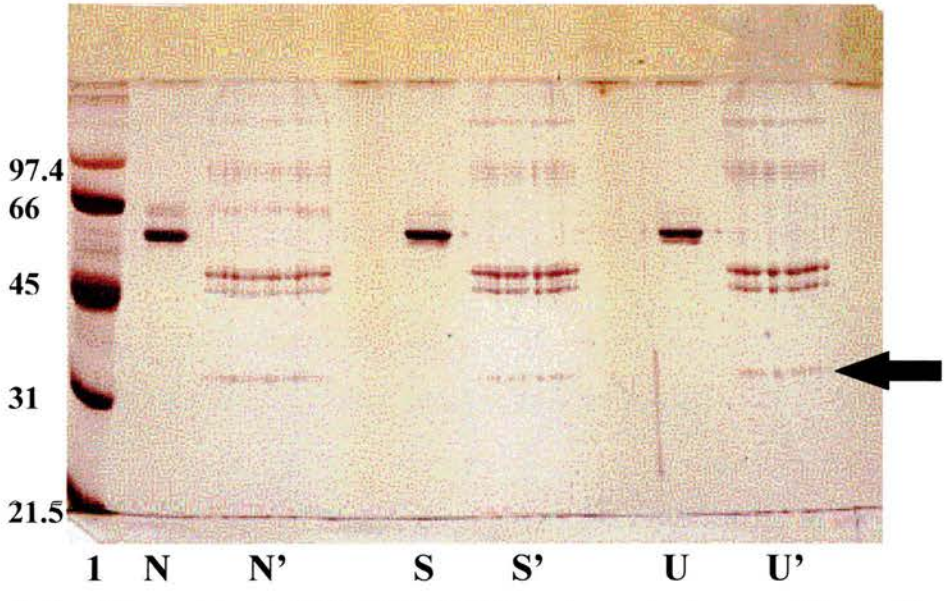

Figure 3.7 - Silver stained 10% SDS-PAGE under reducing conditions of purified Spi1 proteins of haplotypes N, S and U and their respective deglycosylation products N', S' and U'. MW markers (lane 1, kDa) show reduction in size of deglycosylated proteins. Note two deglycosylation products of similar MW for each haplotype (lanes N', S' and U'). PNGase F is a single chain protein with a MW of approximately 32 kDa which can be seen in the three lanes in which deglycosylated proteins have been elecrophoresed (arrow).

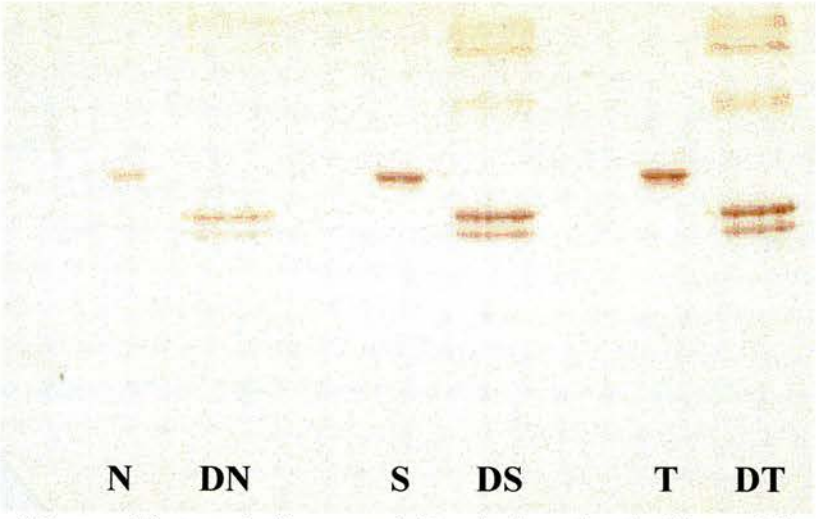

Figure 3.8 - Western blot made from a gel identical to that in Figure 3.7 probed with mAb 1B. Note the weaker positive staining for native Spi1 along with the deglycosylation products in haplotype N (N and DN respectively) compared to that of haplotypes S and T.

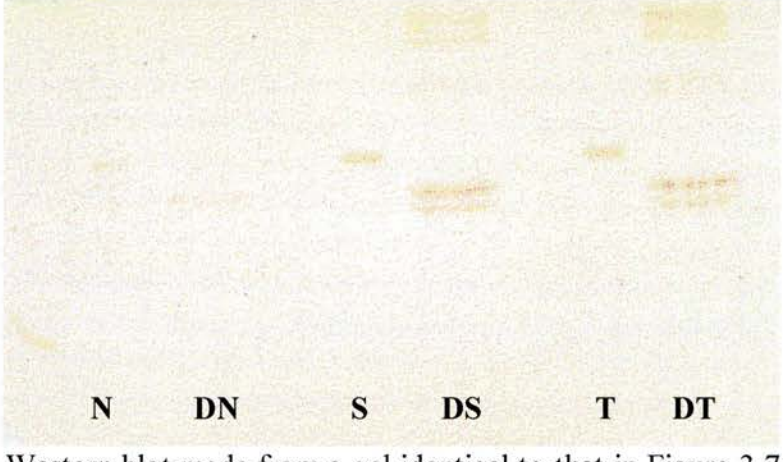

Figure 3.9 - Western blot made from a gel identical to that in Figure 3.7 probed with mAb 1.3.2. The overall intensity of positive staining is less than that of mAb 1B (Figure 3.8) in all haplotypes. The glycosylated (N) and deglycosylated (DN) forms of Spi1 from haplotype N were barely visible.

The purified glycosylated Spi3 from all three haplotypes gave a double band (MWs 64.5 and 61kDa) on 10% SDS-PAGE typical of this isoform (Figure 3.10). Again two main deglycosylation products are evident (MWs 48 and 44kDa) for each haplotype. Examination of the Western blot of the Spi3 isoforms probed with mAb 1B reveals

that both glycosylated and deglycosylated forms in all three haplotypes are recognised (Figure 3.11).

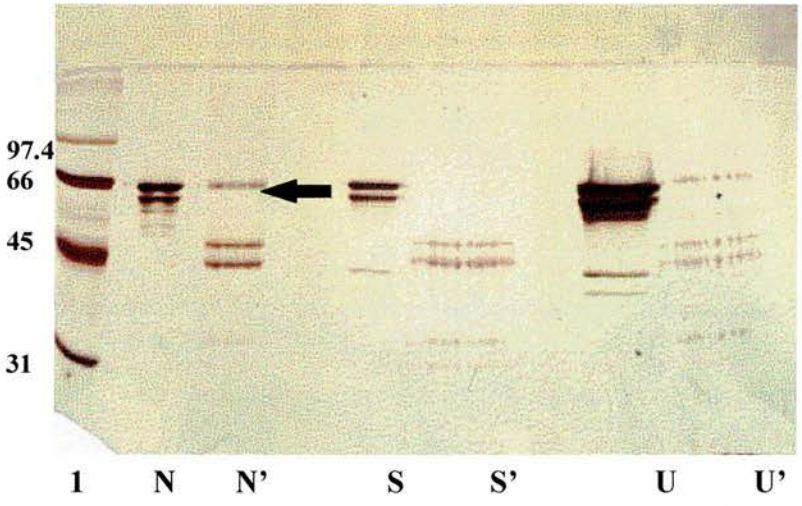

Figure 3.10 - Silver Stained 10% SDS-PAGE under reducing conditions of purified Spi3 proteins of haplotypes N, S and U and their respective deglycosylation products N', S' and U'. Note two deglycosylation products of similar MW for each haplotype (lanes N', S' and U') despite two glycosylated proteins in lanes N, S and U. Also some glycosylated protein remains in sample exposed to PNGase F (arrow).

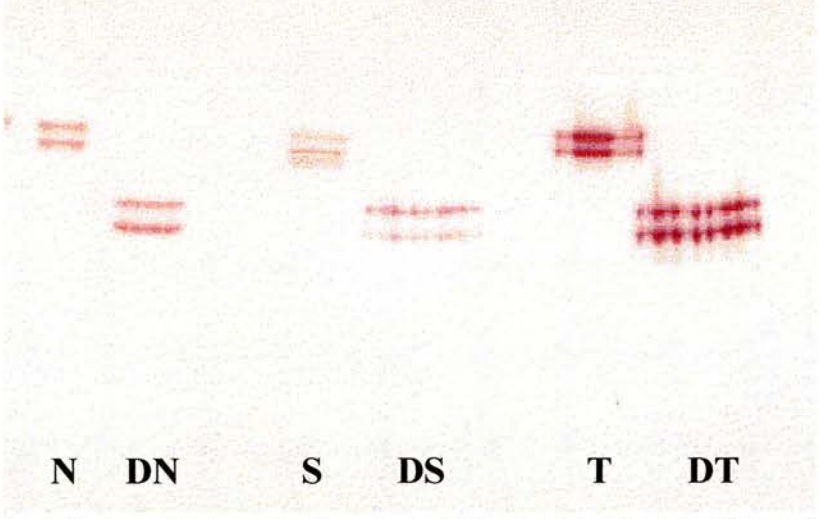

Figure 3.11 - Western blot made from a gel identical to that in Figure 3.10 probed with mAb 1B. Note the presence of a double band in both the glycosylated (N, S and T) and deglycosylated forms (DN, DS and DT) in all three haplotypes examined.

The relative level of staining intensity is similar to that in the blot made from sera under native conditions and probed with mAb 1B (Figure 3.5) in that the U haplotype Spi1 protein stained most intensely followed by N and S respectively. However, the absolute level of immunostaining was greater in the glycosylated Spi3 isoforms of haplotypes N and S when subjected to electrophoresis under reducing conditions (Figure 3.11) compared to native conditions (Figure 3.5). This would suggest that the peptide epitope recognised by mAb 1B is less accessible in the Spi3 isoform of haplotypes N and S probably due to the tertiary structure of the amino acid chain rather than being obstructed by the externally located triantennary CHO side chains. The Western blot of the purified Spi3 isoforms probed with mAb 1.3.2 was devoid of any immunostaining suggesting the peptide epitope recognised by this antibody is probably absent in the Spi3 isoforms of all 8 haplotypes examined (Figure 3.6).

3.4 Discussion

The classical chemical method for purification of API from serum is a laborious four step procedure; (1) chromatography on blue Sepharose to remove albumin, (2) ammonium sulphate precipitation, (3) anion exchange (Mono Q FPLC) chromatography at pH 6.5 and (4) high resolution gel filtration (Superose 12 FPLC) (Pannell *et al* 1974). Despite its complexity the product of this method still suffers from significant contamination with esterase. Equine API can, using immunological means, be specifically and rapidly purified from serum by affinity chromatography in the simple one step procedure described above (Scudamore *et al* 1994) with a purity, as determined by Coomassie stained native PAGE, approaching 100%. Affinity

purification allows large amounts of equine API to be obtained for use as a source of immunising and characterising antigen as well as for use as a standard in ELISAs. The affinity purified API is also the initial step in isolation of individual Spi proteins, requiring one further step involving Mono Q anion exchange chromatography, for more detailed characterisation of Spi specific antibodies.

Using affinity purified API, one polyclonal and two monoclonal antibodies specific for equine API have been successfully raised and characterised. All three antibodies gave consistent, repeatable specificities and sensitivities to the isoforms and haplotypes of equine API in Western blots. Previous studies utilising antibodies raised to equine API were unable to show their exact specificities by any of the accepted methods such as Western blots (Winder *et al* 1989; Milne *et al* 1994c; Scudamore *et al* 1994). The recognition by polyclonal sheep anti-equine API antibody of all Spi proteins with virtually equal sensitivity on Western blots suggests this antibody may be a useful tool; not only for the detection of total API present in tissues by immunohistochemistry but also soluble API in complex biological solutions by sandwich ELISA. The slight variations in the sensitivity of the polyclonal antibody to individual Spi proteins in some haplotypes of API are probably due to variations in Spi protein serum concentrations. API haplotypes F and L have been investigated by video densitometry (Patterson *et al* 1991) to evaluate the serum concentrations of their individual Spi proteins which have been shown to vary between the two haplotypes examined (Table 3.2).

Spi Protein	Serum Concentration of Spi Protein (g/L)	
	Haplotype F	Haplotype L
1	1.6	1.3
2	0.3	0.4
3A	0.34	1.2
3B	0.91	0.6
4	0.8	Not Present

Table 3.2 - Variations in serum concentration of Spi proteins in two haplotypes of equine API (from Patterson et al, 1991).

Both monoclonal antibodies in our study recognise peptide epitopes, suggesting that with respect to their primary amino acid sequence there is a close antigenic relationship between the 4 Spi proteins (mAb 1B recognised all Spi proteins). However, there is also detectable antigenic diversity in that mAb 1.3.2 recognised only Spi1 and 2 and then only in certain haplotypes. The specificity of mAb 1.3.2 supports the suggestion of one study which partially characterised the amino acid sequences of the Spi proteins, including those of the reactive site regions, and found that all four proteins were closely related, but within the four there were two pairs (Spi1 and 2 and Spi3 and 4) which were most strongly related (Patterson *et al* 1991).

The appearance of two protein products from purified Spi1 after PNGase-F treatment suggests that not all of the CHO side chains have been removed during the overnight incubation period. The native Spi3 isoform consists of two isoforms (Figure 3.10) thought to have identical amino acid structures (Patterson *et al* 1991), the more acidic isoform containing two additional terminal sialic acid residues which are thought to be part of an extra complex biantennary CHO side chain (Patterson and Bell, 1986). With Spi3, two products are also seen after overnight incubation with PNGase-F suggesting, again, that incomplete deglycosylation occurs for some reason and also

supporting the suggestion that the difference between Spi3A and Spi3B is in the CHO side chains. This may be due to insufficient exposure time to the enzyme or some form of structural and/or electrostatic hindrance from the tertiary structure of the Spi proteins preventing complete deglycosylation.

In conclusion, the characterisation of the antibodies raised in this study suggests the existence of antigenic relationships and differences between the Spi proteins of equine API due to similarities and differences in the primary amino acid sequences. The polyclonal antibody and mAb 1B have the potential to be useful in further studies in to the immunolocalisation and measurement of total equine API.

Chapter 4

LOCALISATION OF EQUINE ALPHA-1-PROTEINASE INHIBITOR BY IMMUNOHISTOCHEMISTRY

4.1 Introduction

Pulmonary emphysema in horses with neutrophil associated lung diseases, such as chronic obstructive pulmonary disease (COPD), is restricted to a peribronchial pattern of tissue destruction (Kaup *et al* 1990) compared to the relatively extensive centrilobular pattern found in human lung tissue with similar neutrophil associated pulmonary diseases (Creagh and Krausz, 1992) or panacinar destruction with API deficiency (Laurell and Eriksson, 1963). Pulmonary emphysema has been shown to be associated with unregulated NE activity arising from the balance of the proteinase:anti-proteinase equilibrium being shifted in favour of the proteinase side of the equation (Dubin *et al* 1994; Shapiro, 1995). This shift could be due to a decrease in the anti-proteinase screen of the lower respiratory tract (LRT), an increase in the proteolytic burden or a combination of the two. One explanation for the species specific anatomical patterns of emphysema could be a difference in the anti-elastase screen of the lower respiratory tract of horses and humans of which API is the main component (Gadek *et al* 1981a). API found in the pulmonary epithelial lining fluid is derived mainly from the plasma (Milne *et al* 1994c). Plasma API is synthesised in the liver (Carlson *et al* 1988; Hafeez *et al* 1992). However, local production of human API occurs in alveolar epithelial cells (Venembre *et al* 1994), cornea (Twining *et al*

1994), intestinal epithelium (Molmenti *et al* 1993), neutrophils (duBois *et al* 1991) and monocytes and macrophages (Perlmutter *et al* 1985). Investigation of non-pulmonary tissues may be necessary to evaluate the source and distribution of equine API and its role in the anti-elastase screen with regard to the anatomical pattern of equine pulmonary emphysema.

Human API has been extensively investigated in terms of its location in tissues and sites of synthesis by immunohistochemistry (see Chapter 1.7) and detection of API specific mRNA respectively. Development of mRNA probes has been made possible by reference to the known complete amino acid and gene sequences for human API. Interestingly, in humans, despite molecular (Carlson *et al* 1988; Hafeez *et al* 1992) and surgical transplantational studies (Alper *et al* 1980) proving plasma API to be mainly derived from hepatocytes, it has proved difficult to immunolocalise API in the normal Pi MM human liver (Palmer *et al* 1974; Ray and Desmet, 1975; Palmer and Wolfe, 1976; Palmer *et al* 1977; Ray *et al* 1977; Ray and Desmet, 1978; McElrath *et al* 1979) with one exception (Feldmann *et al* 1976). Feldman et al. (1976) found 1-5% of hepatocytes had homogeneous cytoplasmic staining for API by immunohistochemistry.

The localisation of equine API has not been extensively studied. There is only one report of tissue immunolocalisation and this was confined to the liver, lung and peripheral blood leukocytes (Winder *et al* 1989). Two groups have published limited N-terminal amino acid sequences for the equine Spi proteins; one group found all Spi

proteins to be identical for the first 32 amino acid residues examined (Potempa *et al* 1991b), the other found differences between all Spi proteins by residue 20 (Patterson *et al* 1991). No data is available on the gene sequences of the equine Spi proteins and it is therefore not possible at the current time to design primers for use in polymerase chain reaction (Grainger and Madden, 1993) or in situ hybridisation protocols.

The aim of this study was to immunolocalise equine API in several equine tissues using the sheep anti-equine API antibody and mAb 1B in order to compare the distribution pattern with that previously described for humans. Demonstration of species specific differences in API distribution may help to explain the differences in the anatomical patterns of pulmonary emphysema found in the two species in neutrophil associated pulmonary disease.

4.2 Materials and Methods

4.2.1 Choice of Equine Tissues for Immunohistochemistry

In keeping with the aims of this study, equine lung parenchyma and airways were sampled. Since COPD is associated with a large influx of neutrophils into the lung, peripheral blood leukocytes were evaluated for the presence of equine API. Examination of liver was considered essential because it is the site of synthesis in all other species critically examined, (Koj *et al* 1978; Carlson *et al* 1988; Koopman *et al* 1989; Hafeez *et al* 1992). Stomach, pancreas, small intestine and large intestine were sampled because published data in man (Ray and Desmet, 1978; Kittas *et al* 1982; Geboes *et al* 1982) made further comparisons between the two species possible.

4.2.2 Tissue Sampling, Fixation, Storage and Preparation

Tissue and serum samples were obtained from 5 horses at post mortem following clinical diagnoses of:- lameness (2), malignant ovarian tumour (1), behavioural problem (1) and chronic hepatic failure (1). The group comprised 3 mares and 2 geldings, 4-13 years old, with a mean age of 7.6 years. Tissues were removed within 30 minutes of euthanasia, trimmed, and fixed in freshly prepared 4% paraformaldehyde in phosphate buffered saline (PBS) for 24 hours. Samples were then transferred to 70% ethanol and stored at 4°C until embedding in paraffin wax. Sections (4μm) were mounted on TESPA coated slides and stored at 4°C.

Peripheral blood leukocytes were obtained by allowing citrated plasma to sediment under gravity for 30 minutes at room temperature, revealing an upper leukocyte rich plasma layer which was aspirated and centrifuged at 380 x g for 6 minutes. The leukocyte rich pellet was washed four times in Dulbecco's PBS without calcium and magnesium + 0.1% BSA to remove all non-cell associated equine API, followed by resuspension at 5 x 10^6 leukocytes/ml after evaluation by an automated cell counter (System 9000, Serono Baker Diagnostics, Pennsylvania, US). Eighty microlitre samples (5 x 10^6 cells/ml) were centrifuged onto TESPA coated slides (3 minutes at 300 rpm, Shandon Cytospin 3, Southern Instruments Ltd., Runcorn, UK) and fixed in freshly prepared 4% paraformaldehyde in PBS for 45 minutes at 45°C, transferred to 70% ethanol and stored at 4°C until required.

4.2.3 Labelling of Sheep Anti-Equine API Antibody with Horse Radish Peroxidase

Affinity purified sheep anti-ENE 2A antibodies and normal sheep IgG were conjugated to horse radish peroxidase enzyme (HRPO). Their respective RZ values were determined and they were stored as described in Chapter 2.12.1.

4.2.4 Immunohistochemical Localisation of API with Sheep Anti-Equine API

Paraffin embedded equine tissue sections, mounted on TESPA coated slides, were heated to 60°C in a drying oven for 30 minutes to soften the paraffin then dewaxed in xylene and rehydrated to 95% ethanol. Endogenous tissue peroxidase activity was blocked by exposing tissue sections to 3% H_2O_2 in methanol for 20 minutes followed by washing in running tap water for 5 minutes. To improve the accessibility of API antigens to the antibody, an antigen recovery step was incorporated into the procedure at this point.

Initially attempts to improve antigenicity involved exposing sections to 0.1% trypsin in 0.1% CaCl, pH 7.8 at 37°C for 20 minutes followed by two washes in PBS (5 minutes each) which resulted in little or no immunostaining of equine API in the tissues examined by either antibody (data not shown). An alternative, successful, procedure was employed whereby sections were exposed to a 0.01M citrate buffer (Appendix 1), pH 6.0±0.1 at 95-100°C on a heated magnetic stirrer plate for 10 minutes and then allowed to cool at room temperature for a further 10 minutes (Lan *et al* 1995) followed by two washes in PBS (5 minutes each).

Sections were placed in a Sequenza™ Immunostaining Chamber (Shandon Inc., Pittsburgh, USA) and non-specific binding of the sheep anti-equine API antibody was eliminated by incubating sections in 5% normal sheep serum (NSS) in PBS for 30 minutes immediately prior to addition of sheep anti-equine API antibody conjugated to HRPO (1.25μg/ml in 5% NSS/PBS) for 90 minutes. Three washes in PBS (5 minutes each) preceded staining with a DAB peroxidase substrate kit (Vector Laboratories, Peterborough, UK). On appearance of brown colouration (3-5 minutes) sections were washed in running tap water for 2 minutes followed by counterstaining of tissues in Mayer's Haematoxylin and blueing with Scott's Tap Water Replacement (Appendix 1). Sections were initially mounted in Crystal/Mount™ (Biomeda corp., Foster City, CA, USA) then DPX and a coverslip applied. Negative control preparations were treated identically except the sheep anti-equine API:HRPO (RZ=0.700) was substituted with 1.17μg/ml of normal IgG:HRPO (RZ=0.750) in 5% NSS/PBS.

Cytospin preparations were dehydrated in 95% ethanol and, after blocking endogenous tissue peroxidase activity, they were treated identically to the tissue sections, except that the antigen recovery procedure was omitted.

4.2.5 Localisation of Equine API Using mAb 1B and a Biotin Labelled Anti-Mouse IgG Secondary Antibody

To confirm that mAb 1B identified the API haplotypes of the 5 horses from which tissue samples were taken, their sera were subjected to 11.2% native PAGE (with

blue dextran incorporated into the stacking gel to retard albumin migration) and the proteins transferred to Immobilon-P by Western blotting. The Western blot was probed with mAb 1B as described in Chapter 3.2.3.

The initial immunohistochemistry procedure with mAb 1B was identical to that used for the sheep anti-equine API antibody up to and including placing the sections in the Sequenza^{TM} (Shandon Inc., Pittsburgh, USA). However, the blocking solution when using mAb 1B was PBS + 0.5% Tween 80 (PBST80) applied for 30 minutes. For liver sections this was followed by blocking of biotin by a Biotin/Avidin Blocking Kit (Vector Laboratories, Peterborough, UK). All sections were then washed in PBS followed by addition of mAb 1B at 80μg/ml in PBST80 for 90 minutes. Sections were washed in PBS (3 x 5 minutes) followed by addition of goat anti-mouse IgG (Fab specific) antibody conjugated to biotin (Sigma Chemical Co., B 7151) diluted 1/200 in PBST80 for 60 minutes. Sections were washed in PBS (3 x 5 minutes) prior to addition of a preparation of HRPO conjugated to avidin (Vector *Elite* ABC Kit, Vector Laboratories, Peterborough, UK) as per manufacturer's recommendations for 30 minutes. Sections were washed in PBS (3 x 5 minutes) and incubated with 3-amino-9-ethylcarbazole substrate (AEC Peroxidase Substrate Kit, Vector Laboratories, Peterborough, UK) until a red pigment was seen (about 15 minutes) then washed in running tap water (2 minutes). Counterstaining and mounting was identical to that described above. Negative control preparations were treated identically except mAb 1B was replaced with normal mouse IgG_1 (MOPC 31c, Sigma Chemical Co., M 9035) at 80μg/ml in PBST80.

Cytospin preparations were again dehydrated in 95% ethanol and, after blocking endogenous tissue peroxidase activity, they were treated identically to the tissue sections probed with mAb 1B, except that the antigen recovery procedure was omitted.

4.3 Results

4.3.1 Recognition of API Haplotypes by mAb 1B

Monoclonal antibody 1B recognises Spi1 & 2 in all five horses and Spi3 and dimer in four out of five horses (Figure 4.1 & 4.2). The horse in which Spi3 and dimer were not recognised by mAb 1B contained a large amount of protein, probably from haemolysed red blood cells (Figure 4.1). This may have masked recognition of Spi3 and dimer, or mAb 1B may simply not recognise Spi3 in this particular phenotype.

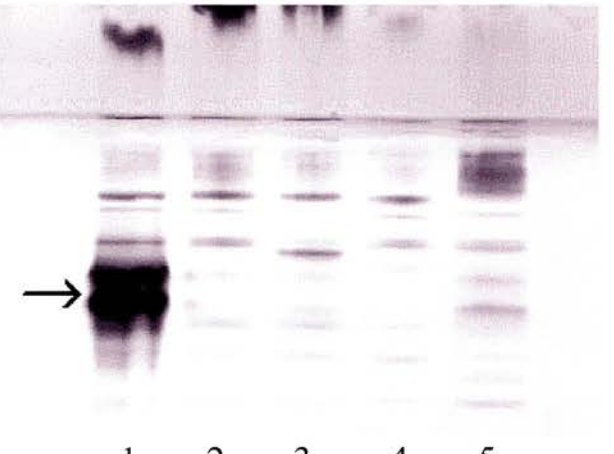

Figure 4.1 - Native 11.2% PAGE of sera from the 5 horses used for immunohistochemistry. Note large amount of protein staining in lane 1 (arrow) which probably results from the haemolysis of red blood cells.

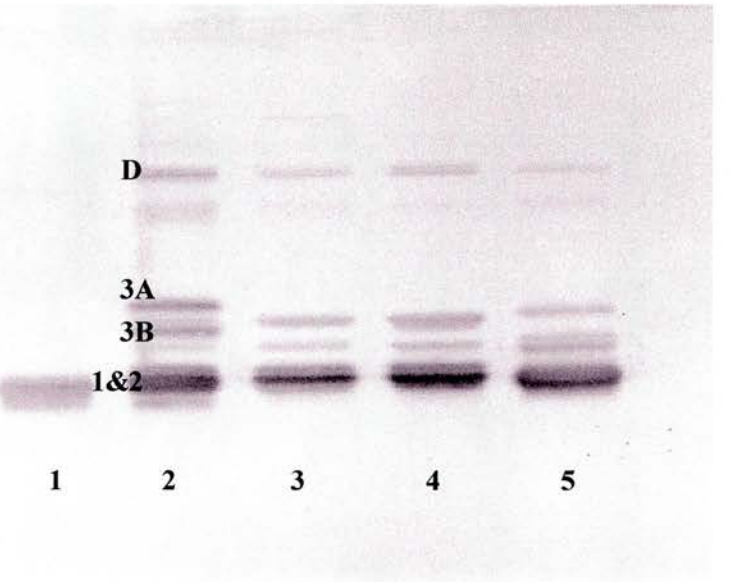

Figure 4.2 - Western blot made from a gel identical to Figure 4.1 probed with mAb 1B. D = dimer, numbers 1&2, 3A and 3B refer to Spi proteins. Note lack of staining of Spi3A, 3B and dimer in lane 1. The horses were euthanased due to; lameness, lanes 1 and 3, behavioural problem, lane 2, ovarian tumour, lane 4 and liver failure, lane 5.

4.3.2 Comparison of Sheep Polyclonal and mAb 1B Immunohistochemistry

Positive staining for equine API in tissues was represented by red pigmentation (AEC) when sections were probed with mAb 1B, and brown pigmentation (DAB) with sheep polyclonal anti-equine API antibody.

Blood vessel contents stain intensely positive for API in all tissues examined with mAb 1B or the sheep anti-equine antibody. Nuclei of cells in all tissues examined (except peripheral blood leukocytes) were devoid of any API when probed with either of the antibodies. Negative control slides, pair matched for each tissue examined in each of the 5 horses, were devoid of any immunostaining

Lung parenchyma and bronchi. Lung parenchyma stained variably for API with both antibodies (Figures 4.3 & 4.4). The cytoplasm of alveolar epithelium varied from unlabelled to intensely positive. Intense staining of multiple cytoplasmic granules is

more prominent with mAb 1B, especially in Type II pneumocytes (Figure 4.5). The most striking difference was the staining of cilia of bronchial columnar epithelium with the sheep anti-equine antibody (Figure 4.6). Whereas, bronchial epithelial staining is absent and only the occasional bronchial epithelial cell has positively staining cytoplasm with mAb 1B (Figure 4.7).

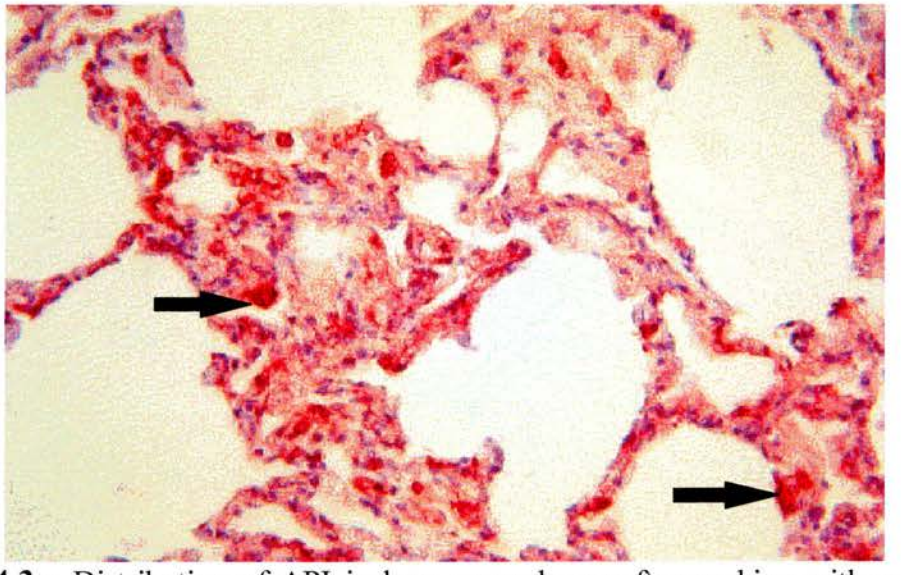

Figure 4.3 - Distribution of API in lung parenchyma after probing with mAb 1B. Note the variability from little or none to intense immunostaining (arrows) in some areas (x 400).

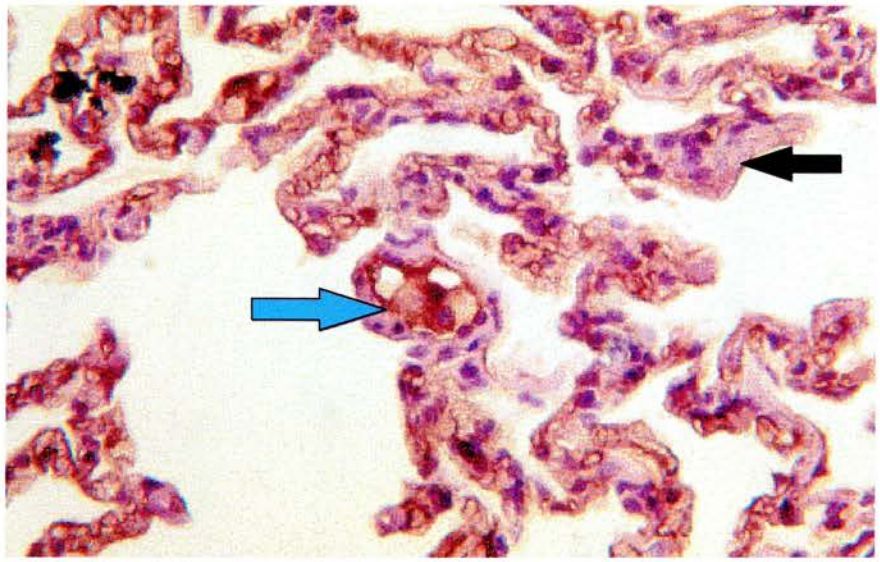

Figure 4.4 - Lung parenchyma probed with the sheep anti-equine API antibody. Very similar pattern to sections stained using mAb 1B, tissues vary from little to no positive staining for API (black arrow) to intense staining in some places. Note capillary contents staining intensely for API (blue arrow). (x 400).

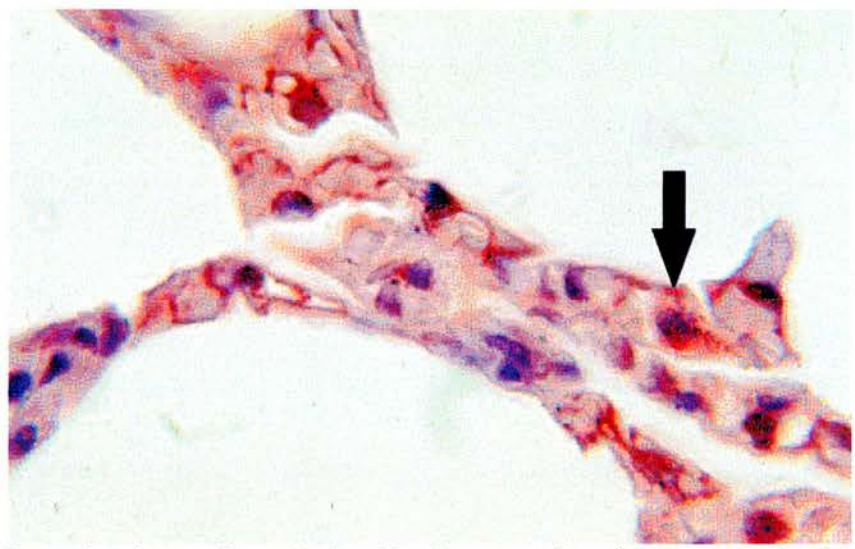

Figure 4.5 - Alveolar wall probed with sheep anti-equine API antibody. Note the intense staining of cytoplasm of a Type II pneumocyte (arrow). (x 1000).

Figure 4.6 - Transverse section of bronchial airway stained using the sheep anti-equine API antibody. Bronchial epithelium cilia stain intensely for API (black arrow) as do blood vessel contents (green arrow) (x 400).

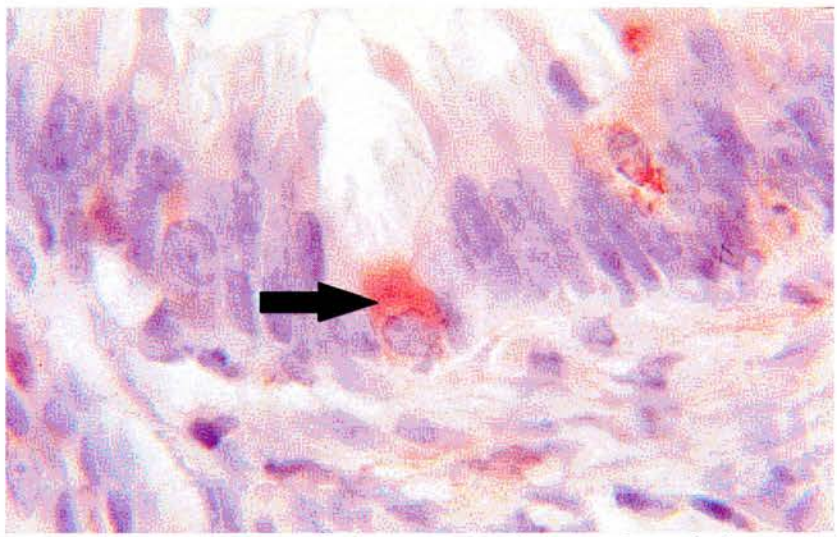

Figure 4.7 - Transverse section of airway epithelium evaluated for API distribution with mAb 1B. Cilia of airway epithelium show no immunostaining with mAb 1B, only the cytoplasm of occasional columnar epithelium cell is positive for API (arrow) (x 1000).

Chondrocytes contained within the lacunae of peribronchial cartilage all have intense cytoplasmic staining with the sheep anti-equine API antibody (Figure 4.8) however this is barely perceptible with mAb 1B (not shown).

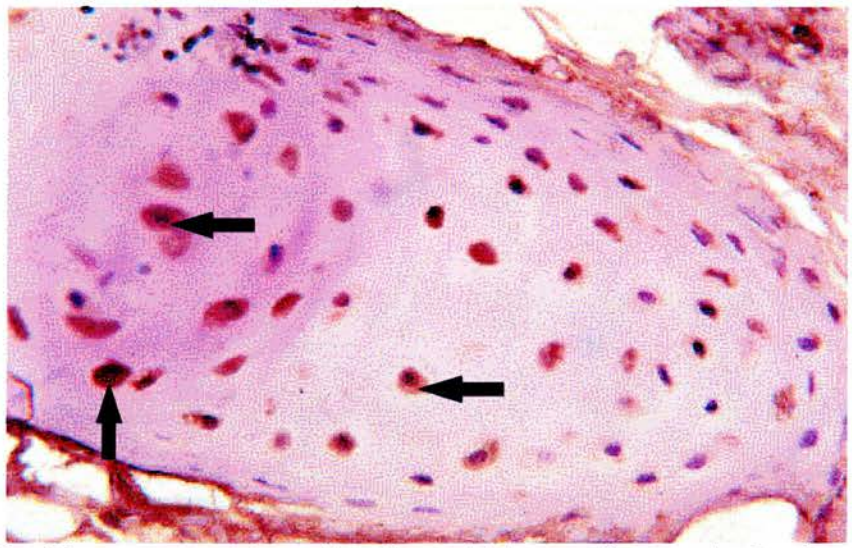

Figure 4.8 - Transverse section of peribronchiolar cartilage stained using the sheep anti-equine API antibody. Cytoplasm of chondrocytes within the lacunae of peribronchiolar cartilage stain intensely for API (arrows) (x 400)

Liver. Cytoplasmic staining of hepatocytes was highly variable with both antibodies. When present, staining was diffuse, with the most intensely staining cells having a centrilobular distribution (Figure 4.9). Some hepatocytes appeared completely devoid of API staining. Intra-cytoplasmic granules of differing size, single or multiple were present in the cytoplasm of some hepatocytes, often in the perinuclear region and grouped together (Figure 4.10). API granules stained more intensely with mAb 1B than with the sheep anti-equine API antibody (Figures 4.10 & 4.11). Intense staining was present in the lumen of the bile ductules where contents were present with both antibodies. The cytoplasm of bile ductule epithelium stained variably for API with the sheep anti-equine API antibody, but intensely with mAb 1B, particularly at the apices of the cells (Figure 4.12- 4.15).

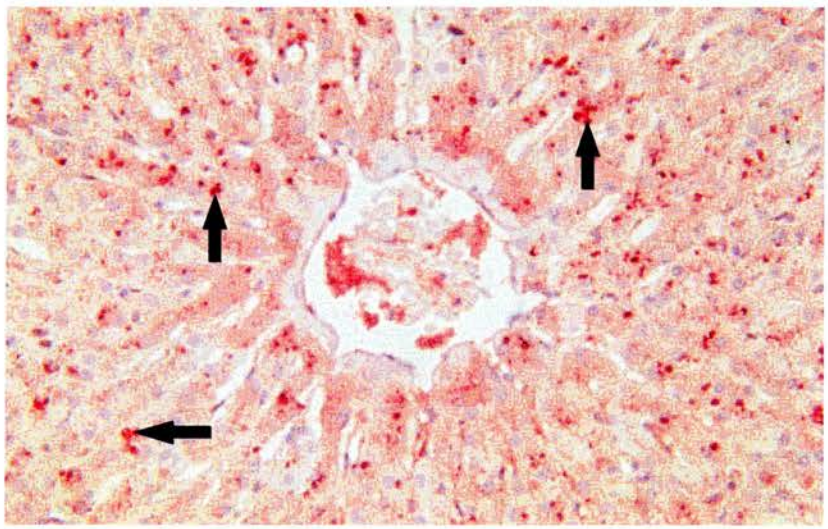

Figure 4.9 - Transverse section of liver through a central vein stained for API using mAb 1B. Note centrilobular distribution of the more diffuse cytoplasmic API immunostaining and the widely distributed granular intracytoplasmic inclusions (arrows) (x 250).

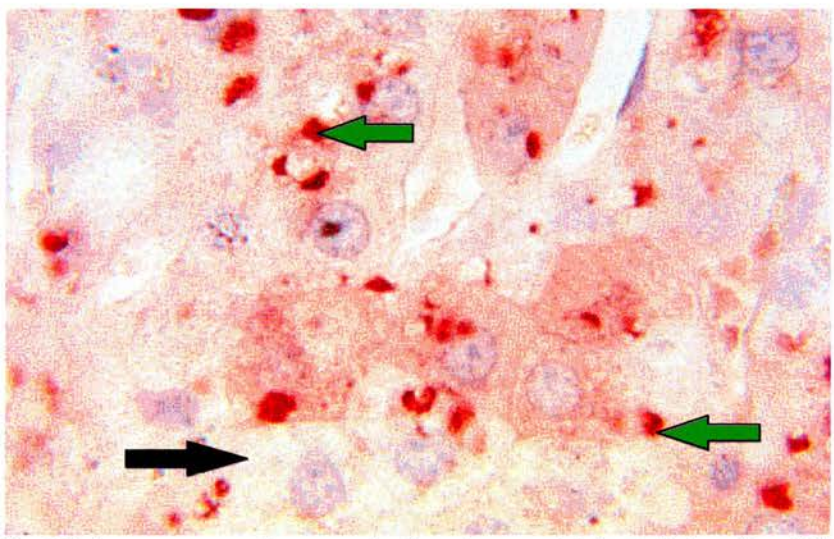

Figure 4.10 - Higher power micrograph showing variable staining for API with mAb 1B. Some cells lack API (black arrow). In many cells API is concentrated in large intracytoplasmic granules (green arrows) (x 1000).

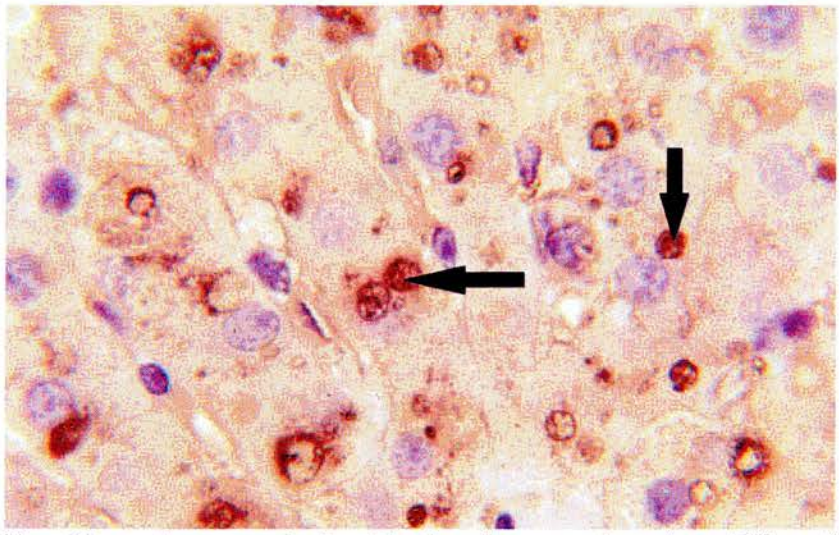

Figure 4.11 - Liver tissue probed with the sheep anti-equine API antibody. API granules (arrows) are less prominent with this antibody and diffuse hepatocyte cytoplasmic staining more consistent between hepatocytes than with mAb 1B (x 1000).

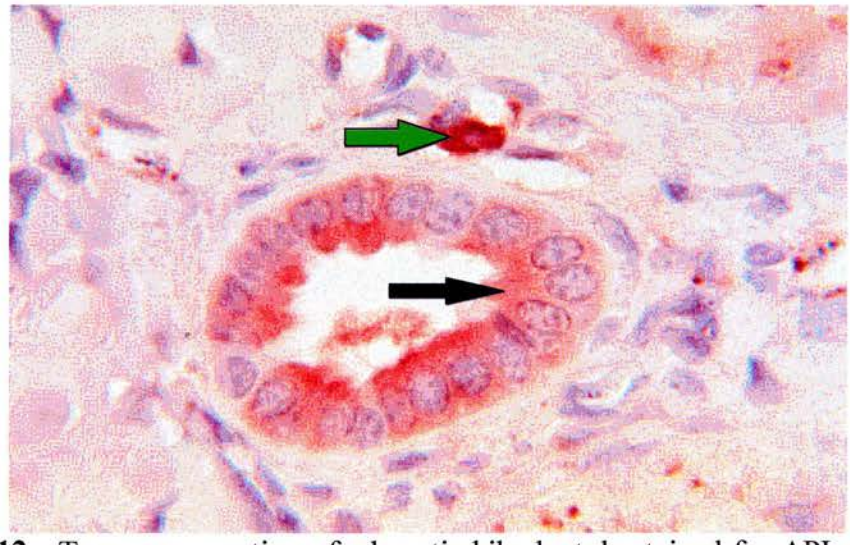

Figure 4.12 - Transverse section of a hepatic bile ductule stained for API using mAb 1B. API is localised in the epithelium (black arrow). A small capillary (green arrow) can also be seen which is intensely stained (x 1000).

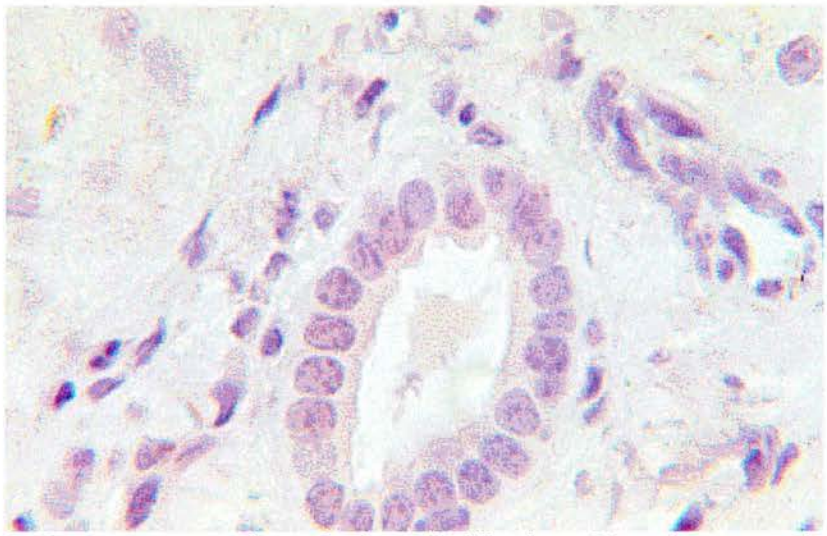

Figure 4.13 - Transverse section of a hepatic bile duct. Negative control preparation in which an identical amount of mouse IgG_1 (MOPC 31c) was substituted for mAb 1B. No specific staining for API is present (x 1000).

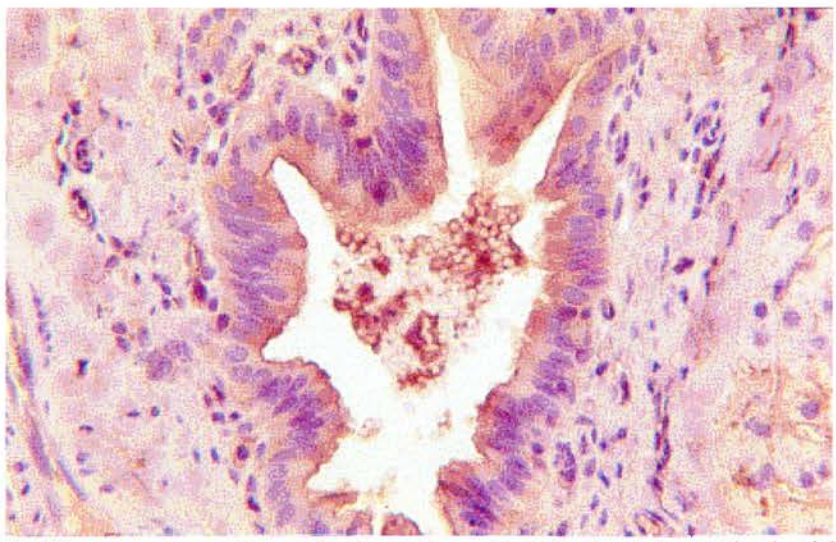

Figure 4.14 - Transverse section of a large hepatic bile ductule probed with the sheep anti-equine API antibody. Note the presence of API in the lumen (x 250).

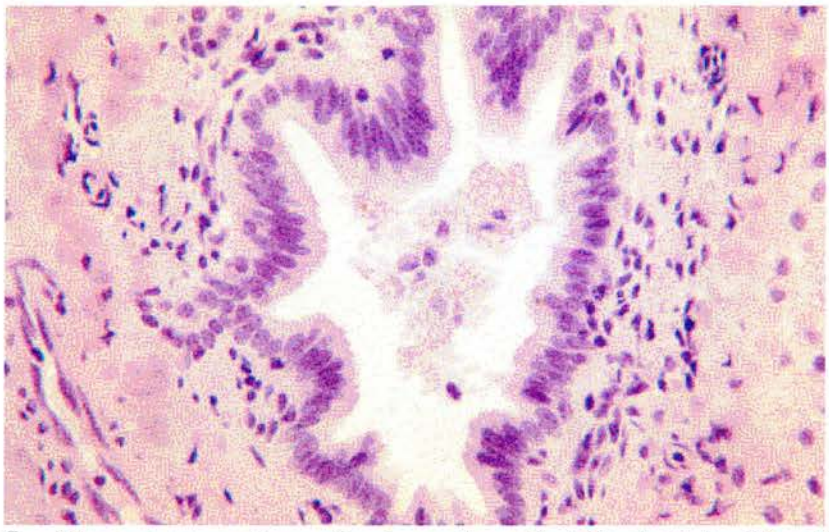

Figure 4.15 - Transverse section of a large hepatic bile ductule. Negative control preparation, the section is devoid of any specific staining (x 250).

Stomach. Luminal mucus, mucous neck cells and lamina propria ground substance are positive for API with both antibodies (Figures 4.16 & 4.17). Using mAb 1B, cytoplasm of peptic, parietal and gastric pit cells is variably positive for API, occasional peptic cells display intense granular staining.

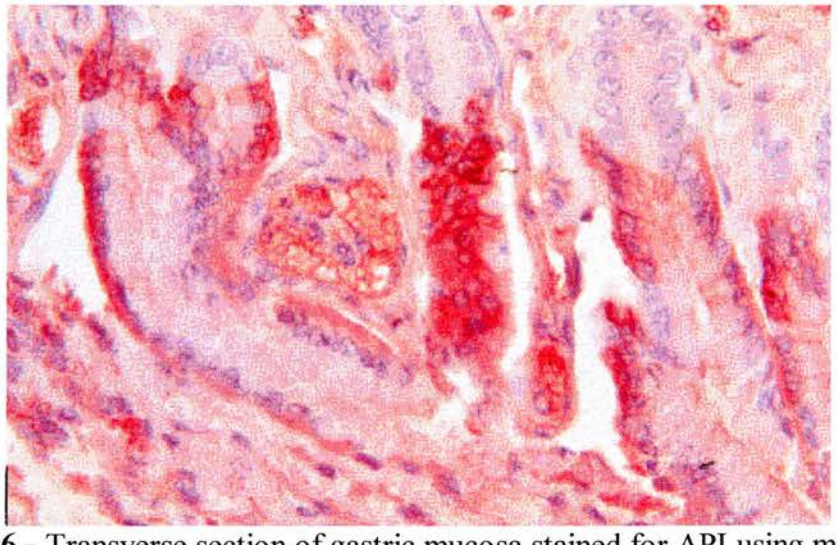

Figure 4.16 - Transverse section of gastric mucosa stained for API using mAb 1B. Note wide variation in intensity of API immunostaining in gastric pits (x 400).

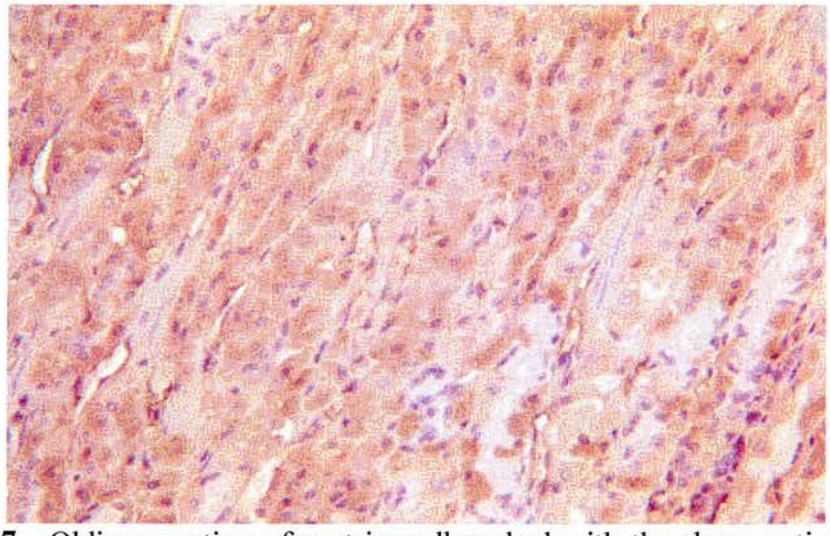

Figure 4.17 - Oblique section of gastric wall probed with the sheep anti-equine API antibody. A more extensive distribution of API is seen throughout the gastric wall cells though some do not stain for API (x 250).

Pancreas. There was intense and uniform localisation of API in the cytoplasm of cells in the Islets of Langerhans with both antibodies (Figures 4.18-4.23). Pancreatic duct contents and the cytoplasm of duct columnar epithelial cells were positive for API with both antibodies, mAb 1B producing a uniformly stippled pattern. Frequent, solitary, very large intensely staining granular inclusions are seen in the basal cytoplasm of these cells (Figures 4.24). Occasional cells throughout the pancreatic parenchyma also had positive cytoplasmic staining especially if in close proximity to large blood vessels.

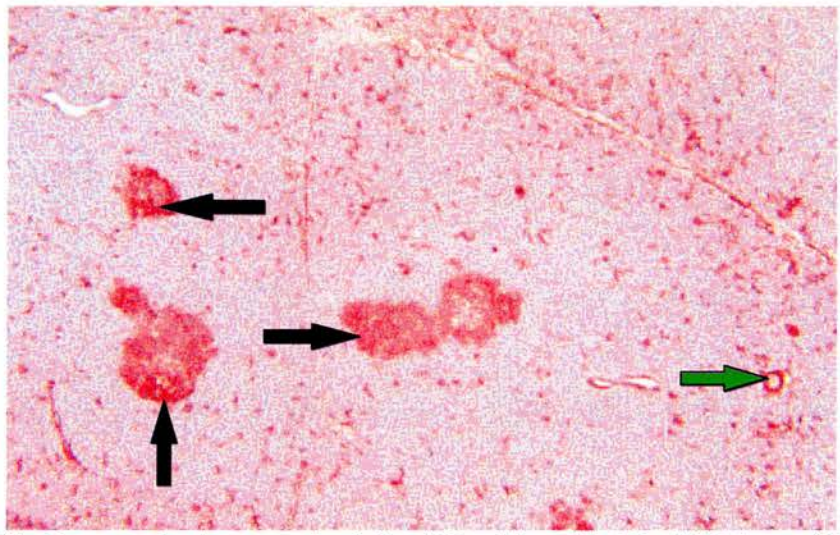

Figure 4.18 - Pancreatic tissue probed for API with mAb 1B. All Islets of Langerhans are intensely stained for API (black arrows) along with blood vessels (green arrows) and occasional parenchymal cells (x 100).

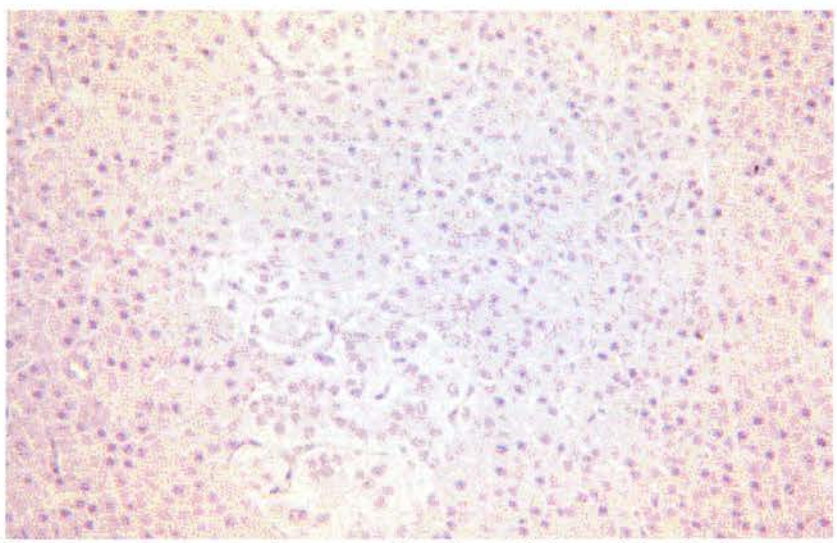

Figure 4.19 - Negative control preparation in which an identical amount of mouse IgG_1 (MOPC 31c) was substituted for mAb 1B. No specific staining is present in the pancreas (x 250).

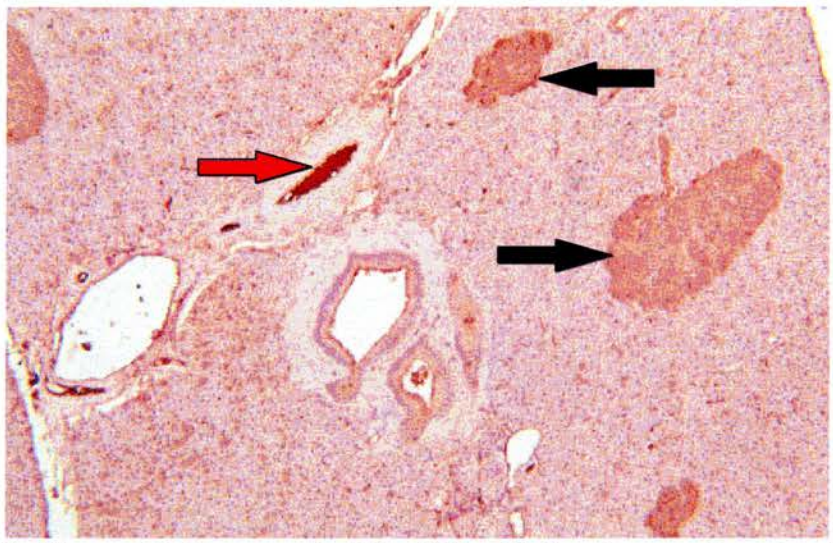

Figure 4.20 - Pancreatic tissue probed for API with the sheep anti-equine API:HRPO antibody. The pattern of immunostaining is identical to that with mAb 1B (see Figure 4.18). Islets of Langerhans (black arrows), blood vessels (red arrows) (x 250).

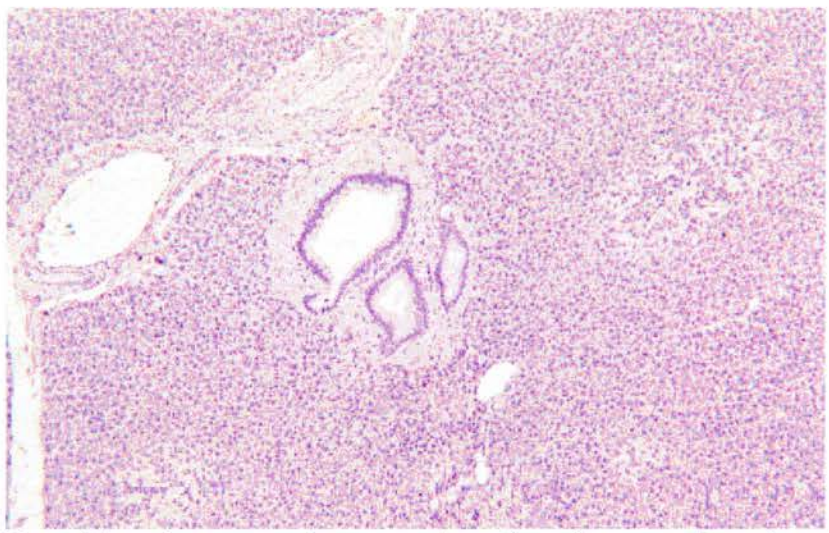

Figure 4.21 - Negative control preparation for sheep anti-equine API:HRPO antibody. The section is devoid of any specific staining (x 250).

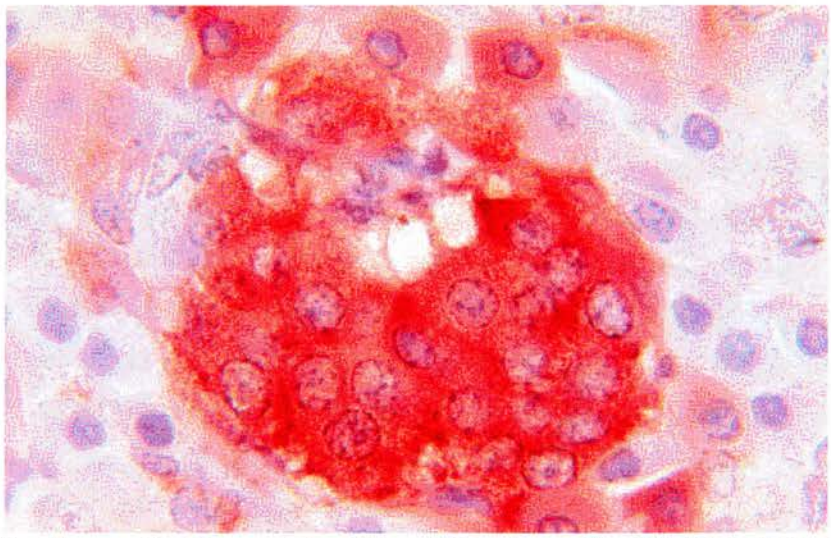

Figure 4.22 - Pancreatic tissue stained for API using mAb 1B. Note intense stippling of the cytoplasm of all Islet of Langerhan cells (x 1000).

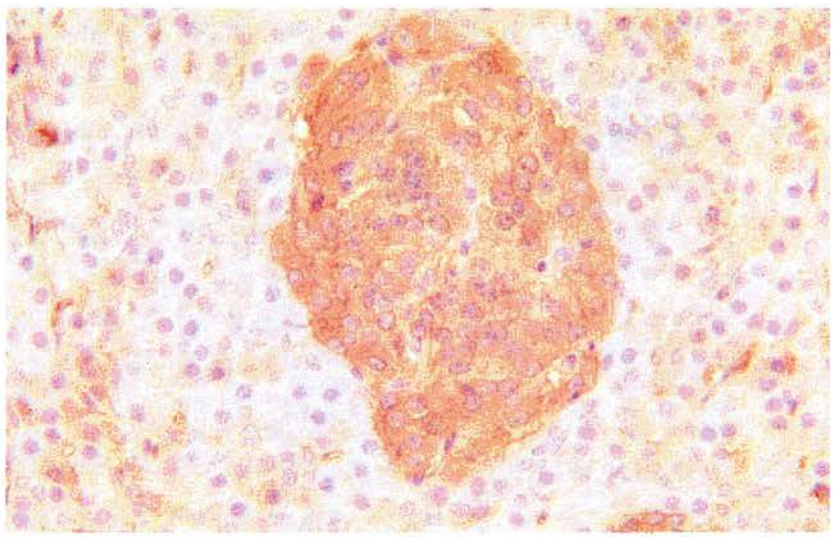

Figure 4.23 - Pancreatic tissue probed with the sheep anti-equine API antibody. The API distribution pattern is similar to that of mAb 1B except it does not have the stippled appearance (x 400).

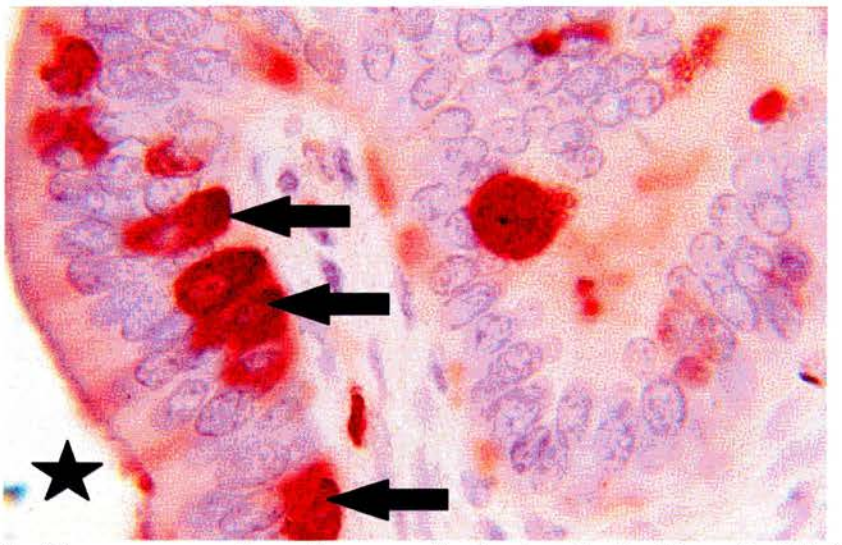

Figure 4.24 - Transverse section of pancreatic duct probed with mAb 1B. Note large intensely staining granules at the base of pancreatic duct columnar epithelial cells (arrows). The star denotes the lumen of the pancreatic duct (x 1000).

Jejunum. The lamina propria along with the cytoplasm of some columnar epithelial cells, most microvilli and occasional submucosal glands are positive for API with both antibodies (Figures 4.25). Goblet cell mucus, however, stained more intensely with the sheep anti-equine API antibody.

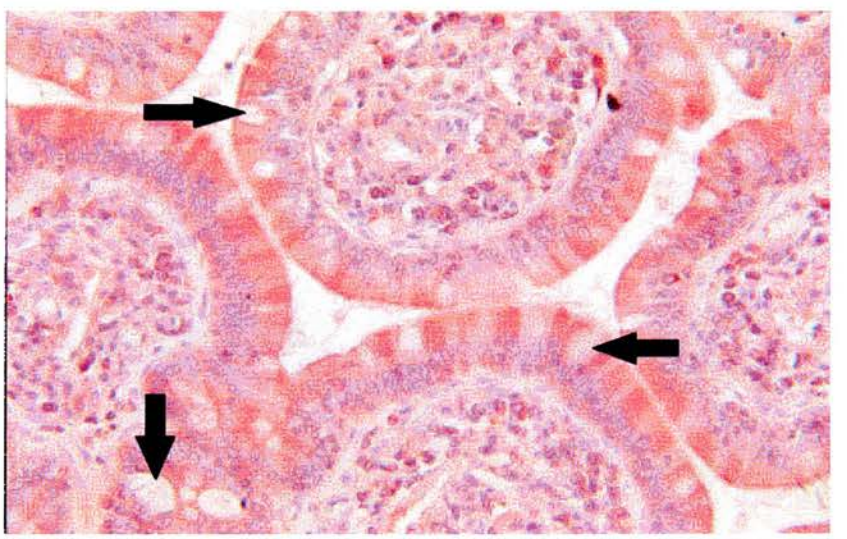

Figure 4.25 - Transverse section of jejunal villi stained for API using mAb 1B. Note the Goblet cell mucus is devoid of API (arrows) (x 250).

Colon. Intense uniform staining of the luminal surface, Goblet cells, plus the mucus and cytoplasm of some columnar epithelial cells was found with the sheep anti-equine API antibody (Figure 4.26 & 4.27). However, with mAb 1B only weak stippled staining of the cytoplasm of some colonic columnar epithelial cells was seen. Goblet cell mucus was rarely positive.

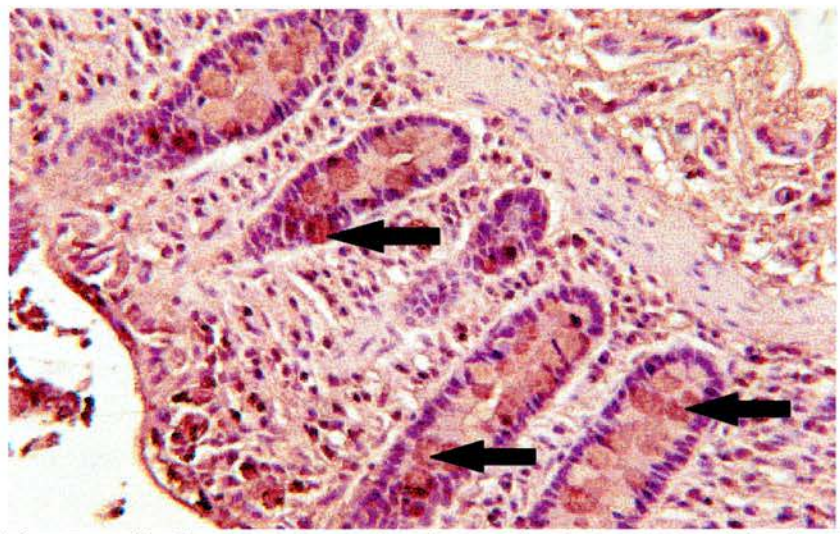

Figure 4.26 - Longitudinal section of colon stained for API with the sheep anti-equine API antibody. Note abundant immunostaining of API of goblet cell mucus (arrows) (x 250).

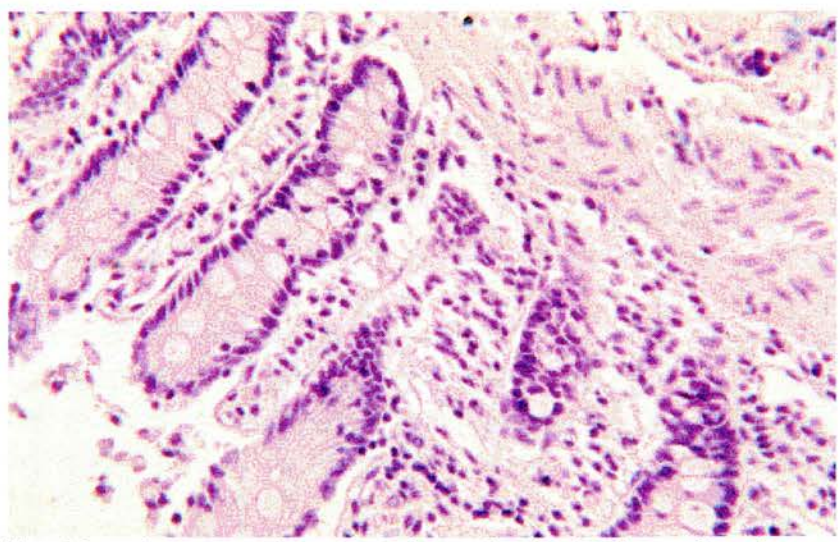

Figure 4.27 - Negative control preparation of colonic tissue for sheep anti-equine API:HRPO antibody. The section is devoid of any specific staining (x 250).

Neuronal Tissue. Sub-bronchial neuronal tissue which was found only on one section appeared to have a sheath of API surrounding individual nerve bundles when probed with mAb 1B (Figure 4.28 & 4.29).

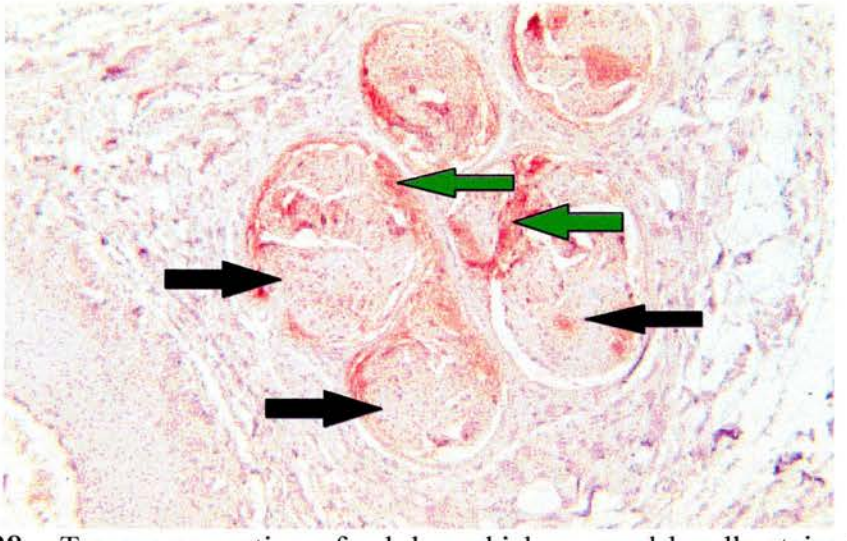

Figure 4.28 - Transverse section of sub-bronchial neuronal bundle stained for API using mAb 1B. Neurones (black arrows) appear bathed in a sheath of API (green arrows) (x 100).

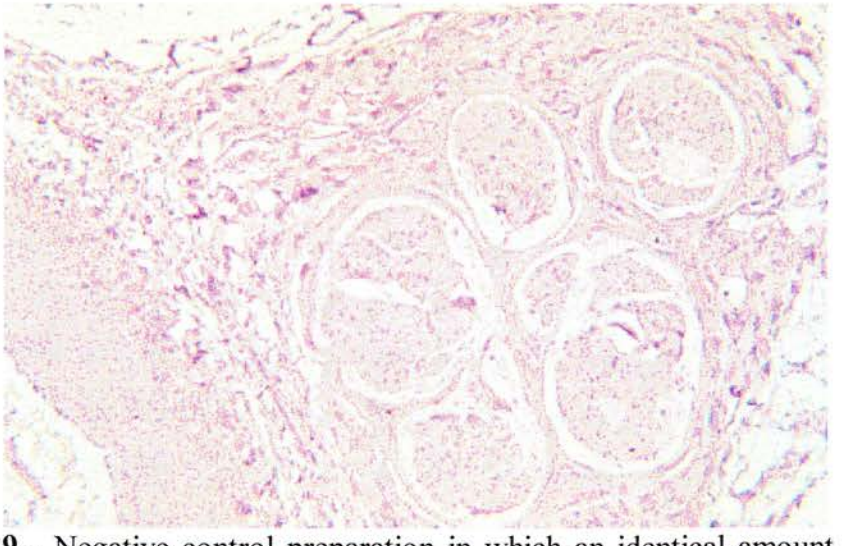

Figure 4.29 - Negative control preparation in which an identical amount of mouse IgG_1 (MOPC 31c) was substituted for mAb 1B as a negative control preparation. The section was devoid of any specific staining (x 100).

Peripheral Blood Leukocytes. Cytosmears of leukocytes were stained with DAB irrespective of whether mAb 1B or sheep anti-equine API:HRPO antibody was used to localise API. Neutrophils had the most intense immunostaining for equine API compared to other leukocytes with a general diffuse cytoplasmic pattern (Figure 4.30 & 4.31). The nuclear membrane appears to stain for equine API but this could be API within the perinuclear cysternum. Monocytes also had faint cytoplasmic and nuclear membrane staining for equine API. The results for peripheral blood leukocytes were identical with both antibodies.

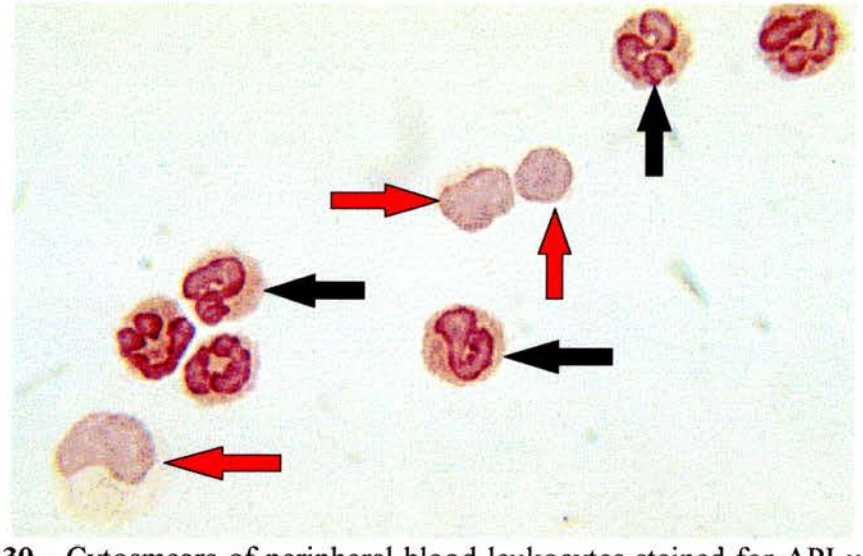

Figure 4.30 - Cytosmears of peripheral blood leukocytes stained for API using mAb 1B. Neutrophils are clearly identifiable by their multilobular nuclei (black arrows). Positive staining for API is represented by brown pigmentation (DAB) and can be seen in the cytoplasm of neutrophils when compared to the negative control cytosmear below (Figure 4.31). Monocytes (red arrows) show very faint staining for API in both their cytoplasm and perinuclear region (x 1000).

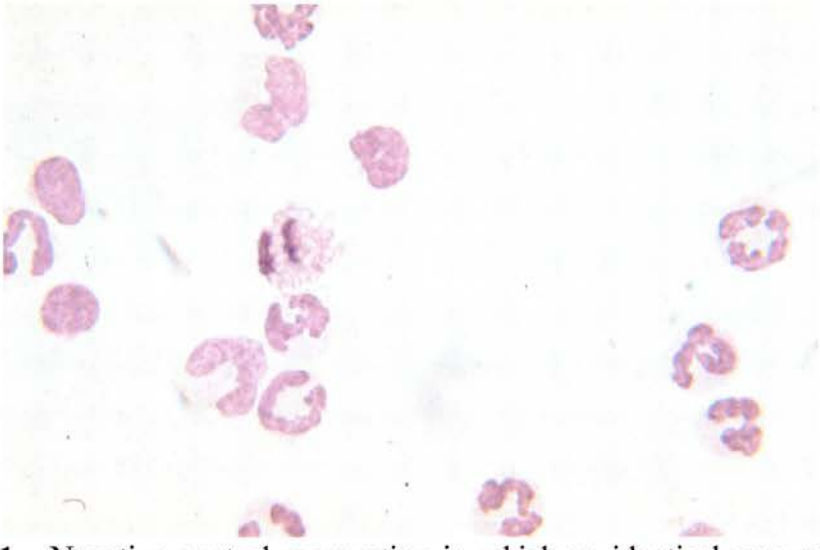

Figure 4.31 - Negative control preparation in which an identical amount of mouse IgG_1 (MOPC 31c) was substituted for mAb 1B. No specific immunostaining for API is present (x 1000).

Summary of Immunolocalisation of API. No difference was seen in the immunostaining pattern or intensity of sections from the horse whose Spi3 was not recognised by mAb 1B on the Western blot suggesting the haemolysed red blood cells may have affected the recognition of Spi3 rather than the antibody being unable to detect it. The horse which was euthanased due to liver failure had extensive hepatic fibrosis, however, where present, hepatocytes had a similar distribution pattern of API as samples from the other four horses (data not shown). Except for this single exception, all tissues from all 5 horses displayed identical distribution patterns for equine API when the same antibody was used. A comparison of the tissue immunostaining patterns of API with the two different antibodies is seen in Table 4.1.

Tissue Sample	Sheep anti-equine API antibody[a]	mAb 1B[a]
Blood vessel contents	++++	++++
Hepatocyte cytoplasm	++++/++	+++/++
Hepatocyte cytoplasmic granules	++	++++
Bile ductule epithelium	++/-	+++
Bronchial epithelium cilia	++++	-
Peribronchial cartilage chondrocytes	+++	+/-
Islets of Langerhans	++++	++++
Pancreatic duct epithelium	+++	++++
Colon Goblet cells	+++	+/-
Colon columnar epithelium	++	+/-
Sub-bronchial neuronal tissue	ND	++

Table 4.1 - Comparison of the Range of immunohistochemical staining intensity[a] in different tissues with the sheep anti-equine API antibody and mAb 1B to equine API. [a] -, no staining; +, ++, +++ and ++++, weak, medium, strong and intense staining, respectively. ND = not determined.

4.4 Discussion

Previous studies of equine API have highlighted its relative complexity in terms of the multiple isoforms (Spi proteins) found in each individual horse (Patterson *et al* 1991; Potempa *et al* 1991b) compared to the single isoform of API present in each human (Gadek and Crystal, 1983; Cox, 1989). Differences in functional properties have also been documented (Fagerhol and Cox, 1981; Patterson *et al* 1991; Potempa *et al* 1991b). This, however, is the first investigation to immunolocalise equine API with antibodies whose specificities have been extensively characterised by Western blotting, both to the affinity purified antigen (equine API) and to the antigen in the presence of other serum proteins. It is also the first time some equine tissues (stomach, pancreas, jejunum and colon) have been investigated for the presence of equine API by any method. It must be borne in mind that immunolocalisation of equine API in a tissue or cell type does not denote synthesis, but a similar

immunostaining pattern to human tissues in which synthesis has been proven by detection of API specific mRNA would suggest the possibility of local production in the horse in the absence of specific equine API mRNA probes.

The boiling citrate antigen recovery procedure (Lan *et al* 1995) was an essential step in the immunolocalisation of equine API in all paraffin embedded tissues examined. Initial attempts at antigen recovery by subjecting sections to trypsin, which has been frequently employed in many other immunolocalisation studies, were unsuccessful suggesting that the equine API antigens might be susceptible to proteolytic cleavage or resistant to exposure by enzymatic digestion of surrounding tissues. Another possibility is that the equine API present in paraformaldehyde fixed, paraffin embedded sections may still possess inhibitory activity thereby binding to the trypsin (a serine proteinase known to be inhibited by native human API) which might result in reduced accessibility to its antigenic epitopes rather than enhancing it.

The sheep anti-equine API antibody and mAb 1B show considerable similarities in the localisation of equine API. The main difference was that mAb 1B gave a more granular staining pattern. The polyclonal sheep anti-equine API antibody will recognise multiple antigenic epitopes on the equine API molecule resulting in more than one antibody molecule binding to each API molecule leading to an enhancement of detected signal. The monoclonal antibody, however, will probably only recognise a single antigenic epitope which may only occur once on each API molecule. The latter will result in poorer detection of diffusely distributed API compared to granular

accumulations of protein. This may explain why mAb 1B appears to give a more granular pattern than the sheep anti-equine API antibody in many tissues.

The most striking difference between the staining patterns of the sheep anti-equine API antibody and mAb 1B was found in the lungs. Intense staining of the cilia of bronchial columnar epithelium with the sheep anti-equine API antibody (Figure 4.6) is completely absent with mAb 1B. This could be explained by a variation in antibody sensitivity as described above, however, the difference is so great that the sheep anti-equine API antibody may detect a serpin not recognised by mAb 1B. Similar disparities were seen when the chondrocytes contained within the lacunae of peribronchial cartilage (Figure 4.8) and mucus within goblet cells of the colon (Figure 4.26) were stained and compared.

Type II pneumocytes, which were intensely positive for equine API in this study (Figure 4.5), have been shown in humans (using the A549 cell line which is used as a model of human type II pneumocytes), by Northern blot analysis the presence of human API specific transcripts (Venembre *et al* 1994) which is taken to indicate protein synthesis. This suggests that synthesis of equine API from type II pneumocytes might also occur in the horse. It has also been shown that human bronchial epithelial cells synthesise and secrete API (Cichy *et al* 1997). However, equine API was not detected in bronchial epithelial cells by immunostaining, only in the cilia of the cells and then only by the sheep anti-equine API antibody (Figure 4.6). The intracellular distribution of API in human bronchial epithelium has not been

investigated. Nevertheless, biochemical studies show that human bronchial epithelial cells synthesise API with glycosylation patterns which differ from API synthesised by human HepG2 hepatocytes. The latter has a mixture of 3 isoforms of variably bi and tri-antennary glycosylated proteins whereas that from human bronchial derived epithelium contained all tri or tetra-antennary CHO side chains (Cichy *et al* 1997). If an analogous situation exists in the horse and the CHO side chains are significantly different from those of serum derived API (which was used to characterise both antibodies used in this study) it may reduce the sensitivity of immunodetection by physically or electrostatically blocking antibody access. This could explain why the sheep anti-equine antibody with its multi-epitope specificity immunolocalises equine API in the cilia of bronchial epithelium but mAb 1B does not.

Equine hepatocytes appear to be the major source of plasma API in the horse due to the extensive immunostaining and this is supported by the data from human (Carlson *et al* 1988; Hafeez *et al* 1992), rat (Koj *et al* 1978) and mouse studies (Koopman *et al* 1989). Where present, equine API immunostaining in hepatocytes has two distinct patterns, diffuse and granular. The diffuse cytoplasmic staining may be indicative of synthesis in the endoplasmic reticulum with the granules in the perinuclear region suggestive of post-translational modification and packing in the Golgi complex prior to export from the cell.

When compared with previous studies of human liver (Palmer *et al* 1974; Ray and Desmet, 1975; Palmer and Wolfe, 1976; Palmer *et al* 1977; Ray *et al* 1977; Ray and

Desmet, 1978; McElrath *et al* 1979), equine hepatocytes show extensive immunostaining for API. This indicates either a difference in the absolute levels of hepatic API between the two species or some significant variation in methodology.

Circulating plasma concentration of equine API (3.5g/l) are high when compared to human plasma API (1.3g/l). This may be due to a greater amount of synthesis per hepatocyte, hence a greater amount of API per cell. This in turn may result in a level of API in the horse above the threshold detectable by immunohistochemical means, whereas in the human the level may be below this threshold, possibly due to rapid synthesis and secretion of API in the human hepatocyte, so that immunodetection is not possible.

Human API immunolocalisation studies of liver biopsies used a variety of tissue fixatives and rarely employed an antigen recovery step in the procedure, which, if present, usually consisted of exposure to trypsin, the results of which this study found disappointing. The use of a more sensitive technique, paraformaldehyde fixation producing less cross linking of proteins combined with a better antigen recovery procedure was found to give superior results.

The intense immunostaining of the Islets of Langerhans in the pancreas suggests a large amount of equine API is present which has been previously proposed to have an anti-enzymatic function (Ordonez *et al* 1985). It has been suggested that this is to protect the delicate protein hormones insulin and glucagon produced in these cells

from the actions of proteolytic enzymes such as trypsin (a serine proteinase inhibited by API) which is produced in the surrounding parenchyma along with many other proteolytic enzymes for use in intestinal digestion (Bendayan and Ito, 1979; Gauldie *et al* 1980). The pancreatic ducts which transport the proteolytic enzymes to the digestive tract have columnar epithelial cells that contain large amounts of API, some in granular form (Figure 4.24), presumably as protection against proteolytic enzyme damage also.

Human pancreatic studies only show immunostaining of API in the peripheral cells of the Islets of Langerhans (Ray *et al* 1977; Ray and Desmet, 1978) which is again probably a difference in methodology rather that a true difference in distribution.

Although the horse has a relative over abundance of API proteins compared to humans, the similarity of the tissue distribution in the two species suggests that the sites of local production of API in humans are likely to be present in the horse too. However, until specific mRNA probes are available for equine API this will be difficult to verify.

In conclusion this study has found that, with the exception of the liver, there is little or no difference in the tissue distribution of immunolocalised API between horses and humans in the tissues so far examined. This suggests that the specific location of API in equine tissues is, in itself, unlikely to be responsible for the differences observed in

the anatomical patterns of emphysema encountered in horses and humans in neutrophil associated pulmonary diseases.

Chapter 5

IMMUNOLOCALISATION AND QUANTITATION OF ELASTASE IN EQUINE PERIPHERAL BLOOD NEUTROPHILS

5.1 Introduction

Equine peripheral blood neutrophils are believed to contain significantly less NE (0.4pg/cell) (Dubin and Koj, 1986) than human neutrophils (1.1pg/cell) (Tetley *et al* 1989). The lower elastase content of equine neutrophils, combined with the greater plasma concentration of API in the horse (Patterson *et al* 1991) (compared to humans), may result in a more efficient anti-elastase screen/less potent proteolytic capacity in the lower respiratory tract of horses compared to humans.

The NE content of peripheral blood neutrophils have been estimated by different techniques in the two species; cell extracts from purified neutrophils were evaluated for elastinolytic activity in horses (Dubin and Koj, 1986) versus a specific competitive ELISA for HNE, independent of elastase activity, for humans (Tetley *et al* 1989). However, we have shown immunocytochemically that API, the major inhibitor of NE in the lower respiratory tract (Gadek *et al* 1981a), is present in the equine neutrophil (Chapter 4.3.2). It has also been shown that API found in human peripheral blood neutrophils, where it can be synthesised by the mature cells, is capable of inhibiting the HNE released from the cells (duBois *et al* 1991). Other studies have identified an elastase inhibitor confined to the cytoplasm of equine neutrophils, horse leukocyte elastase inhibitor (HLEI), present at a concentration of 0.5pg/cell (Dubin and Koj,

1986) which interacts with equine neutrophil elastase with a $K_{ass} = 10^7\ M^{-1}s^{-1}$, $K_i = 10^{10}$ M, forming inhibitory complexes which are stable on SDS-PAGE (Dubin and Hauck, 1981; Dubin *et al* 1985; Dubin *et al* 1986).

The presence of these two inhibitors, API and HLEI, suggests that the evaluation of the NE content of equine peripheral blood neutrophils by a method dependent upon elastinolytic activity of cell extracts may underestimate the true concentration of ENE as a proportion may be inhibited during the extraction process and thus not be detected.

Equine neutrophil elastase consists of two major proteinases ENE 2A and ENE 2B (Chapter 1.4), which have been shown to have different N-terminal amino acid sequences, molecular weights, elastinolytic activities, isoelectric points and relative proportions in the neutrophil (Dubin and Koj, 1986; Dubin *et al* 1994). ENE 2A is reported to be three times more abundant than ENE 2B (Dubin and Koj, 1986) and recent studies of equine API:ENE interactions and functions have used ENE 2A (Scudamore *et al* 1993; Pemberton *et al* 1993). Due to the greater potential yield of ENE 2A during purification procedures and its prior use in proteinase:anti-proteinase studies ENE 2A has formed the main focus of this study.

The aim of this study was to evaluate the concentration and distribution of ENE 2A in equine peripheral blood neutrophils in order to assess the proteinase side of the proteinase:antiproteinase equilibrium in the horse. Specifically to:

i) purify equine neutrophil elastase 2A,

ii) using immunological techniques develop a specific method of detecting and measuring ENE 2A in complex biological samples whether in the free or complexed state,

iii) show the presence and distribution pattern of ENE 2A in equine peripheral blood neutrophils by light microscopy and

iv) evaluate the relationship between the distribution of ENE 2A and that of intracellular API in the neutrophil.

5.2 Materials and Methods

5.2.1 Production of a Sheep Polyclonal Antibody to ENE 2A

Purification of ENE 2A. ENE 2A was extracted from equine peripheral blood neutrophils by a modification of a previously published method (Dubin *et al* 1976). Six litres of equine whole blood were collected immediately post mortem (from one adult gelding) into sterile bottles containing 100ml 1.36% ethylene-diamino-tetraacetic acid (EDTA) in PBS per litre of blood. Free sedimentation under gravity for 45 minutes revealed an upper leukocyte rich plasma layer which was aspirated and centrifuged at 600 x g, 4°C for 15 minutes to pellet the leukocytes. From this stage onwards all samples were kept on ice. The supernatant was carefully removed without disturbing the cell pellet and replaced with 36ml of sterile water for 20 seconds, to remove any remaining red blood cells by hypotonic lysis. This was immediately followed by addition of 12ml 3.6% NaCl to restore the solution to physiological osmolality. The resultant cell suspension was centrifuged at 700 x g, 4°C for 10

minutes followed by careful removal of the dark red supernatant. The cell pellet was resuspended in 60ml of PBS, centrifuged at 700 x g, 4°C for 10 minutes, followed by removal of the supernatant and resuspension of the cell pellet in 240ml of 0.2M sucrose solution with heparin (150iu/ml). The suspension was then split into 10ml aliquots which were homogenised (Ultra-Turrax T25, narrow probe, Fisher Scientific, Leicestershire, UK) for 20 seconds to disrupt leukocytes. If the suspensions were still very viscous samples were homogenised for a further 20 seconds. Aliquots were pooled and centrifuged at 2000 x g, 4°C for 25 minutes to pellet cell debris and leave the cytoplasmic granules (which contain ENE) in suspension. The supernatant was removed and centrifuged at 20000 x g, 4°C for 30 minutes to pellet granules. Granule pellets were pooled and washed twice in 10ml of 0.3M sucrose and were centrifuged each time at 20000 x g, 4°C for 30 minutes. The granule pellet could be stored at -70°C at this point before being subjected to further extraction processes, if required, or used immediately.

The granule pellet was suspended in 30ml of 1M NaCl, 0.05% Triton X 100 and stirred overnight at 4°C in a glass universal to disrupt granules, causing release of their enzymatic contents. The mixture was diluted 1:6 with 50mM 2-[N-Morpholino] ethanesulphonic acid (MES) buffer, pH 6.0, 0.1% polyoxyethylene 23 lauryl ether (BRIJ 35) to reduce the salt concentration (≈0.14M). The mixture was centrifuged at 20000 x g, 4°C for 30 minutes to obtain a clear supernatant containing elastase which could be stored in 50ml aliquots at -20°C before the next stage in purification, if required.

The above supernatant, containing ENE 2A and 2B was subjected ion-exchange chromatography using FPLC and a twin buffer system:

Buffer A 50mM MES, pH 6.0, 0.1%BRIJ

Buffer B 50mM MES, pH 6.0, 1M NaCl, 0.1% BRIJ

A Resource-S (strong cation) column (Pharmacia, Uppsala, Sweden) was equilibrated with 95% Buffer A + 5% Buffer B at 4ml/minute. The above granule extract was diluted 1:1 with Buffer A and loaded on to the Resource-S column followed by equilibration with the original buffer mixture. To elute the bound proteins a gradient of 5-60% Buffer B was applied to the loaded Resource-S column over 20 minutes. The elastases elute as two major separate peaks between 20-30% Buffer B with ENE 2A eluting first and having a peak three times larger than that of ENE 2B. Fractions were collected on the basis of UV absorbance and temporarily stored at 4°C.

To purify the ENE 2A, the eluate from the Resource-S column corresponding to this fraction was diluted with an equal volume of Buffer A and applied to a Mono-S (strong cation, with smaller bead size that Resource-S) column (Pharmacia, Uppsala, Sweden) equilibrated with 90% Buffer A + 10% Buffer B at 1ml/minute. Following loading of the sample the buffer gradient was then altered from 10-60% Buffer B over 40 minutes with the ENE 2A eluting as the highest detectable peak at around 20% Buffer B. Fractions were collected into separate test tubes every 60 seconds and those corresponding to the large protein peak were subjected to 12% SDS-PAGE and silver stained to assess purity. Samples were stored at -50°C.

For the final purification step, the fractions most enriched for ENE 2A were subjected to molecular weight exclusion chromatography by HPLC on a Supadex 75 column (Pharmacia, Uppsala, Sweden) equilibrated with PBS, 0.5M NaCl, 0.1% BRIJ, 0.05% sodium azide. Fractions were collected upon visualisation of UV absorbance and subjected to 12% SDS-PAGE followed by staining with Coomassie brilliant blue (R-250) to assess protein purity.

Antigen Inoculation Procedure. To prepare antibodies which would recognise ENE 2A in the complexed as well in the native free state, 50μg of purified ENE 2A was incubated with 100μg of affinity purified equine API in PBS for 30 minutes at room temperature (21°C) to allow complex formation. The solution of ENE 2A:API complex was combined with adjuvant and inoculated using an identical protocol to that for raising the polyclonal sheep anti-equine API antibody (Chapter 3.2.1).

Purification of Polyclonal Sheep Anti-ENE 2A Antibody. Sheep polyclonal antibodies were purified by affinity chromatography as described for sheep anti-equine API antibodies (Chapter 3.2.1). However, in this case, 1mg of purified ENE 2A was used as the ligand (see Chapter 2.8). The affinity purified anti-ENE 2A antibodies were desalted, quantified and stored as previously described (Chapters 2.4.1 and 3.2.1).

5.2.2 Isolation of Equine Peripheral Blood Leukocytes

Equine peripheral blood neutrophils were isolated by modification of a previously published method using a discontinuous Percoll gradient and centrifugation which

separates cells according to their density (Haslett *et al* 1985). All blood was taken by jugular venipucture and anticoagulated with 1ml of 3.8% sodium citrate (Phoenix Pharmaceuticals Ltd., Gloucester, UK) per 10ml of blood. Free sedimentation under gravity for 30 minutes at room temperature revealed an upper leukocyte rich plasma layer which was aspirated and centrifuged at 380g for 6 minutes. Platelet-rich plasma supernatant was decanted and centrifuged at 2400g for 20 minutes to prepare platelet poor plasma (PPP). The initial leukocyte rich pellet, obtained typically from 80ml of whole blood, was resuspended in 2ml PPP in a 15ml polystyrene tube. This was underlayered initially with 2ml of a 42% solution of 90% Percoll (90% Percoll, 10% sterile 0.9% NaCl)/58% PPP followed by 2ml of 52% x 90% Percoll/48% PPP layer. The resultant discontinuous gradient comprising leukocytes in PPP, 42% Percoll and 52% Percoll from top to bottom was centrifuged at 255g for 12 minutes at 21°C. Two tight bands were resolved, cytospins prepared from these revealed that the mononuclear cells were retained in the PPP/42% Percoll interface and neutrophils in the 52%/42% interface. Neutrophils harvested from this layer were washed sequentially in PPP, 4 times in Dulbecco's PBS without calcium and magnesium/0.1% BSA and once in Dulbecco's PBS with calcium and 0.1% BSA prior to resuspension in the latter. All procedures were performed at room temperature (21°C). Purity was assessed by differential count of Diff-Quik™ stained cytospins and cells counted by an automated cell counter (System 9000, Serono Baker Diagnostics, Pennsylvania, US).

5.2.3 Characterisation of the Sheep Anti-ENE 2A Antibody

The specificity of the sheep anti-ENE 2A antibody was evaluated by probing Western blots of ENE 2A. Neutrophil extracts were prepared from pellets of 126 x 10^6 Percoll purified equine peripheral blood neutrophils by the addition of 200µl 3M NaCl and 400µl SDS-PAGE reducing or non-reducing sample buffer (Chapter 2.5.1) and repeated freeze thawing (5 cycles). The sample containing reducing buffer was heated to 95°C. Both samples were centrifuged (20000g, 30min. at 4°C) to remove DNA and other cell debris and neutrophil extracts were stored at -70°C until required.

Purified ENE 2A (0.5µg per lane), neutrophil extract (20µl of the above preparation per lane), API:ENE 2A complex (1.0µg:0.5µg respectively per lane) and API (1.0µg per lane) were separated by 10% SDS-PAGE under reducing and non-reducing conditions to give several identical gels. One gel (reducing conditions) was stained with Coomassie brilliant blue (R-250) to visualise all proteins present. Proteins from similar gels (under reducing conditions or non-reducing conditions) were transferred to Immobilon-P by semi-dry Western blotting (Chapter 2.7). Membranes were probed with the either affinity purified sheep anti-ENE 2A antibody (see below) or with mAb 1B (anti-equine API) as described in Chapter 3.2.3.

For the sheep anti-ENE 2A antibody, non-specific binding on Western blots was blocked with 3% dried skimmed milk (J.S. Sainsbury, London, UK) in PBS (3%MPBS) for 60 minutes prior to addition of the primary antibody at 5µg/ml (in 3%MPBS) for 60 minutes. Following 3 washes in PBS + 0.05% Tween 20 and one in

PBS respectively, for 5 minutes each, donkey anti-sheep IgG:HRPO conjugate (Sigma Chemical Co., A 3415) at 1/4000 dilution in 3% MPBS was added for 60 minutes. An identical wash procedure as before was then followed by addition of DAB peroxidase substrate kit (Vector Laboratories, Peterborough, UK) as per manufacturer's instructions to visualise conjugated antibody. On appearance of protein bands the membranes were washed in 3-4 changes of dH_2O, photographed and subsequently dried on filter paper prior to storage in a light proof container. An identical procedure was performed on an identical control Western blot except the primary antibody was substituted with 5μg/ml of normal sheep IgG.

5.2.4 Assessment of Proteolytic Activity of Purified Antigens

Proteolytic activity of purified ENE 2A, neutrophil extract, API:ENE 2A complex and API was assessed by incorporating 20mg azocasein per 10mls of resolving gel solution in 10% SDS-PAGE under non-reducing conditions (Horie *et al* 1984) as described in Chapter 2.6.4. Sample loading was as described above in 5.2.3., proteolysis being seen as a clear band on the blue background of the gel.

5.2.5 Immunolocalisation of ENE 2A in Peripheral Blood Leukocytes Using Sheep Anti-ENE 2A Antibody

Cytospins of peripheral blood leukocytes were prepared, fixed and stored as described in Chapter 2.15. Staining consisted of an identical procedure to that described in Chapter 4.2.4 except the blocking solution was 5% normal rabbit serum in PBS (5%NRS/PBS). The primary anti-ENE 2A antibody used in this study was diluted to

1.25 μg/ml in 5%NRS/PBS, the conjugate (rabbit anti-sheep IgG:HRPO, Dako Ltd., High Wycombe, Bucks, UK) was diluted 1/400 in 5%NRS/PBS and the peroxidase substrate was AEC (Vector Laboratories, Peterborough, UK) added as per manufacturer's instructions. On appearance of red coloration (about 9 minutes) sections were washed, counterstained and mounted as described in Chapter 4.2.4. Negative control preparations were treated identically except the sheep anti-ENE 2A was substituted with an identical concentration of normal sheep IgG (Sigma Chemical Co., I 5131), 1.25μg/ml in 5% NRS/PBS.

5.2.6 Colocalisation of ENE 2A and API in Peripheral Blood Leukocytes by Dual Immunofluorescence

Conjugation of sheep anti-ENE 2A antibody to Cy3™ and sheep anti-equine API antibody to fluorescein isothiocyanate (FITC) was performed as described in Chapter 2.12.2. Cytospins identical to those used for immunocytochemistry above were rehydrated and exposed to DAB 4mg/10ml PBS, 50μl H_2O_2 (30% w/w) for 15 minutes to quench endogenous tissue fluorescence followed by three 5 minute washes in dH_2O and three in PBS respectively. Non-specific binding of antibodies was blocked by addition of 5%NSS/PBS for 30 minutes. The primary antibodies were added together in a total volume of 80μl per slide at final concentrations of 5μg/ml and 7μg/ml for sheep anti-ENE 2A antibody and sheep anti-equine API antibody respectively in 5%NSS/PBS for 90 minutes. Following three 5 minute washes in PBS cytospins were mounted in Vectashield (Vector Laboratories, Peterborough, UK), a coverslip applied and sealed with nail varnish.

Negative control cytospins were treated identically except the primary antibodies were substituted for two preparations of normal sheep IgG conjugated to Cy3 and FITC with their respective concentrations altered for their fluorochrome to protein ratios.

5.2.7 Determination of the Amount of ENE 2A in an Equine Peripheral Blood Neutrophil by a Specific Sandwich ELISA

Development of a Specific Sandwich ELISA to Measure ENE 2A. Micro-ELISA plate wells (Dynex Technologies, Billingshurst, West Sussex, UK) were coated with 50µl of affinity purified sheep anti-ENE (1.5µg/ml) in 0.1M carbonate/bicarbonate buffer (Appendix 1). After incubation at 4°C overnight, plates were washed 6 times with 0.9% NaCl containing 0.05% Tween 20 (0.9% NaCl-T20). Samples (in duplicate) and standards (in triplicate) were diluted in 5%MPBS containing 0.05% Tween 20 (5%MPBST20), 50µl of each standard or sample were added per well. Purified ENE 2A was used as a standard over the range 0.5 to 4.0 ng/ml. After addition of samples and standards, plates were incubated at room temperature (21°C) for 1 hour followed by washing 6 times in 0.9% NaCl-T20. Fifty microlitres of affinity purified sheep anti-ENE 2A conjugated to HRPO (1µg/ml in 5%MPBST20, see Chapter 2.12.1) was added per well and plates incubated for a further hour at room temperature followed by a final 6 washes in 0.9% NaCl-T20. To initiate colour development 50µl of TMB (Dynex Technologies, Billingshurst, West Sussex, UK) was added per well as a soluble substrate for the HRPO enzyme and the reaction terminated by addition of 25µl of 0.18M H_2SO_4. The colour intensity was measured at

490nm on a MR7000 ELISA plate reader (Dynex Technologies, Billingshurst, West Sussex).

To evaluate the intra ELISA plate variation one row of blanks and three rows of standards were applied to a plate as usual. To the remaining 64 wells 50µl of an ENE 2A solution, from a large single quality control batch diluted to be in the middle of the standard curve, were added. The plate was developed in the usual manner and the consistency of the OD values examined by coefficient of variance (see below). Inter ELISA plate variations were assessed by adding 50µl of the above quality control ENE 2A solution to two wells in every ELISA plate used to determine the concentration of ENE 2A in various samples. This was also analysed by coefficient of variance using the following equation:

Coefficient of variance = standard deviation/mean x 100

Evaluation of Sheep Anti-ENE 2A Antibody Recognition of Complexed ENE by ELISA. ENE 2A in the free and complexed state was subjected to the ELISA described above. To 200ng of purified ENE 2A (in 100µl PBS) affinity purified API (800ng in 100µl PBS) was added and incubated for 30 minutes at room temperature to allow formation of ENE 2A:API complex. An identical sample of 200ng of purified ENE 2A was diluted with 100µl of PBS to bring the concentration of free ENE 2A equal to that of the complexed ENE 2A (200ng/200µl PBS). Each sample was further diluted and applied to the above ELISA in triplicate at concentrations of 0.5-4.0ng/ml of ENE 2A.

Evaluation of the Amount of ENE 2A per Equine Peripheral Blood Neutrophil.

Peripheral blood leukocytes from 11 horses (6 mares, 4 geldings and 1 stallion, median age 10 years, range 5-19 years) were purified as above and resuspended at $5x10^6$cells/ml. Diff-Quik™ stained cytospins were used to determine the percentage of neutrophils in each of the Percoll purified preparations, 500 cell being counted from random high power (x 1000) fields (Chapter 2.14). One millilitre samples were taken from each sample and stored at -70°C then subjected to 5 rapid freeze/thaw cycles to induce cell lysis. DNA and cell debris were removed by centrifugation (13800 x g, 10 minutes at 4°C). ENE 2A content in each supernatant was estimated using the ELISA. Supernatants were diluted between 1/250 to 1/32000 in 5%MPBST20. To determine the amount of ENE 2A per equine neutrophil the following equation was used :

ENE 2A per neutrophil = ELISA value (ng/ml)/
(No. of cells per ml in supernatant x percentage neutrophils)

5.2.8 Evaluation of the Amount of Equine API per Equine Peripheral Blood Neutrophil

The polyclonal sheep anti-equine API raised and characterised in this study (Chapter 3.2.) was used in an equine API specific ELISA with minor modifications to the previously described protocol (Scudamore *et al* 1994). Essentially the method was identical to that described above (5.2.7) except that the coating antibody (sheep anti-equine API antibody) concentration was 1μg/ml, and the blocking and diluting solution consisted of 4.5% BSA in PBS containing 0.05% Tween 20. The conjugate

was sheep anti-equine API antibody:HRPO prepared as described in Chapter 2.12.1 and diluted to 1μg/ml. Affinity purified API was used to create the standard curve.

The same samples used to determine ENE 2A content per equine peripheral blood neutrophil were used to determine the amount of equine API except the samples were diluted from 1/10 to 1/1280 times in 4.5%BSA/PBS/T20.

5.3 Results

5.3.1 Purification of ENE 2A

ENE 2A was successfully purified from equine peripheral blood neutrophils using a combination of ion-exchange and MW exclusion chromatography. Subsequent to ion-exchange chromatography high and low MW contaminants were still present in the sample when examined by silver stained 12% SDS-PAGE under reducing conditions (Figure 5.1). Molecular weight exclusion chromatography resolved these contaminants into three distinct peaks on HPLC (Figure 5.2). The result was ENE 2A which was 100% pure as determined by Coomassie brilliant blue (R-250) stained 12% SDS-PAGE (Figure 5.3).

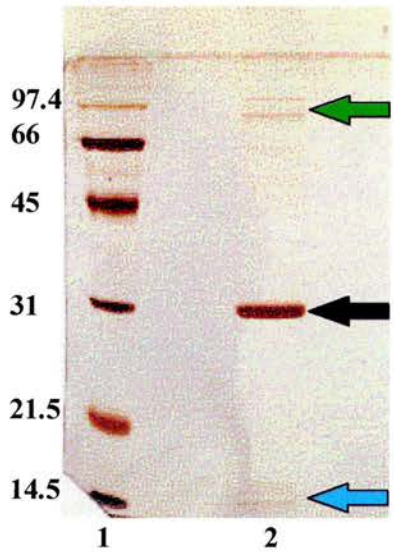

Figure 5.1 - Determination of protein purity by silver stained 12% SDS-PAGE under reducing conditions after ion-exchange chromatography. Lane 1 contains MW markers (kDa), lane 2 the purified protein. Note, ENE 2A (black arrow) is the major protein with less intensely stained high MW (green arrow) and low MW (blue arrow) contaminants.

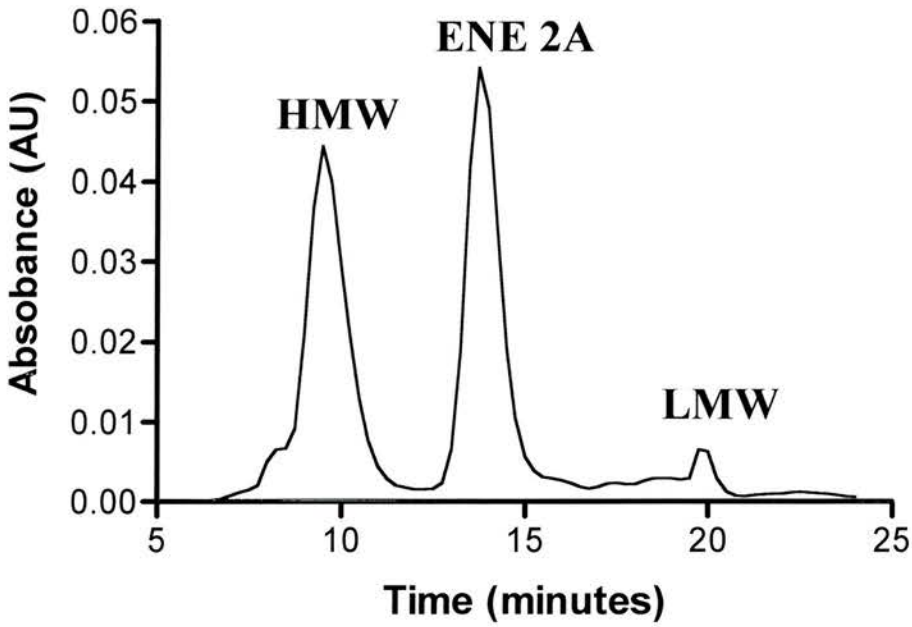

Figure 5.2 - Graph of absorbance versus time for MW exclusion chromatography showing the resolution of the three distinct peaks of high (HMW) and low MW (LMW) contaminants along with that of ENE 2A which enabled further purification of the ENE 2A.

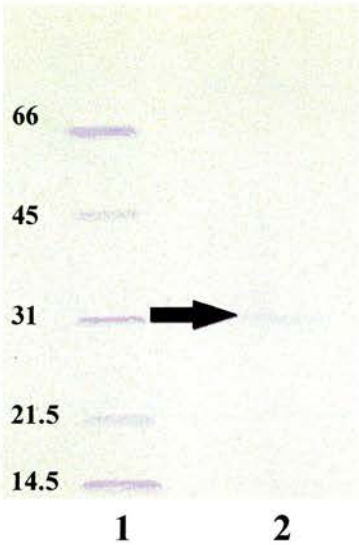

Figure 5.3 - Determination of ENE 2A purity by Coomassie brilliant blue (R-250) stained 12% SDS-PAGE after MW exclusion chromatography. Lane 1, MW markers. Lane 2, ENE 2A (arrow).

5.3.2 Isolation of Equine Peripheral Blood Neutrophils

The purity of equine neutrophils isolated on a Percoll discontinuous gradient from 11 different horses was 96.5% ± 3.0 (mean ± SD, n =11, Figure 5.4).

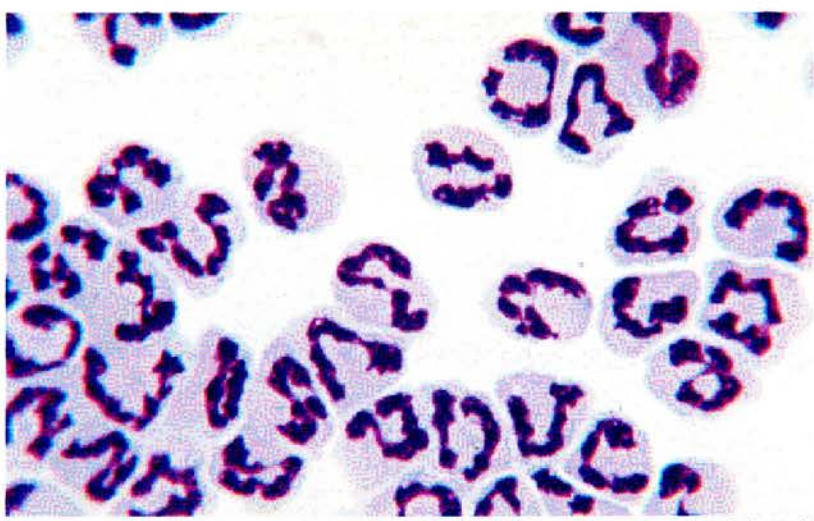

Figure 5.4 - Percoll purified peripheral blood leukocytes stained with Diff-Quik™ (x 1000).

5.3.3 Characterisation of the Sheep Anti-ENE 2A Antibody

Purified API isoforms and API:ENE 2A complex had MW from 57-69kDa and 79-108kDa respectively (Figure 5.5), these ranges reflecting the differing MW of API isoforms Spi1, 2 and 3 present in the affinity purified equine API (Patterson *et al* 1991).

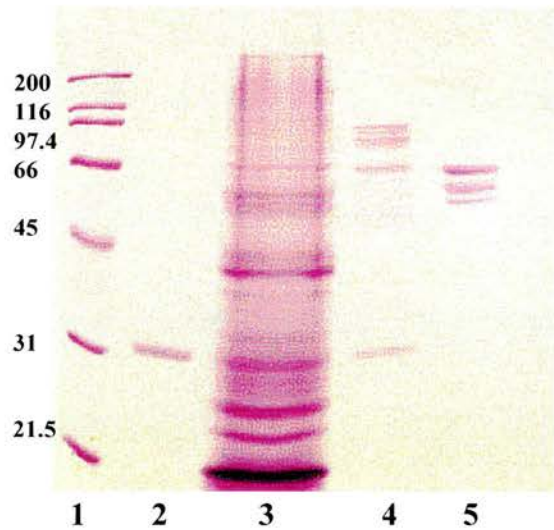

Figure 5.5 - Coomassie brilliant blue (R-250) stained 10% SDS-PAGE under reducing conditions comprising; MW markers, purified ENE 2A (0.5µg), neutrophil extract (20µl), API:ENE 2A complex (1.0µg:0.5µg respectively) and affinity purified API (1.0µg) in lanes 1-5 respectively. Note the higher MW of API:ENE 2A complex (lane 4) compared to native API (lane 5).

Western blots showed no cross reactivity between sheep anti-ENE 2A and sheep anti-equine API antibodies to API or ENE 2A respectively in the reduced (Figure 5.6) or non-reduced state (data not shown), yet both clearly recognised API:ENE complex (Figure 5.6). Further scrutiny of the specificity of the sheep anti-ENE 2A antibody reveals that it identifies its target protein in the neutrophil extract despite the presence of other proteins and also a band of 55kDa MW (Figure 5.6). On the azocasein gel (Figure 5.7) proteolysis was absent in the equivalent area of this 55kDa MW band yet

all uncomplexed ENE 2A found in lanes 2, 3 and 4 displayed proteolytic activity which suggests the 55kDa band may be an elastase:inhibitor complex. Several studies have reported the intracellular serpin, horse leukocyte elastase inhibitor (HLEI), to have a MW of 35.2kDa (Dubin, 1977; Dubin and Koj, 1986; Potempa *et al* 1988), hence the 55kDa band may represent ENE 2A complexed with HLEI. This complex is stable in the presence of SDS under reducing conditions (Figure 5.6) unlike that of secretory leukocyte proteinase inhibitor (SLPI), found in human neutrophils (Sallenave *et al* 1997a), which can form complexes with HNE.

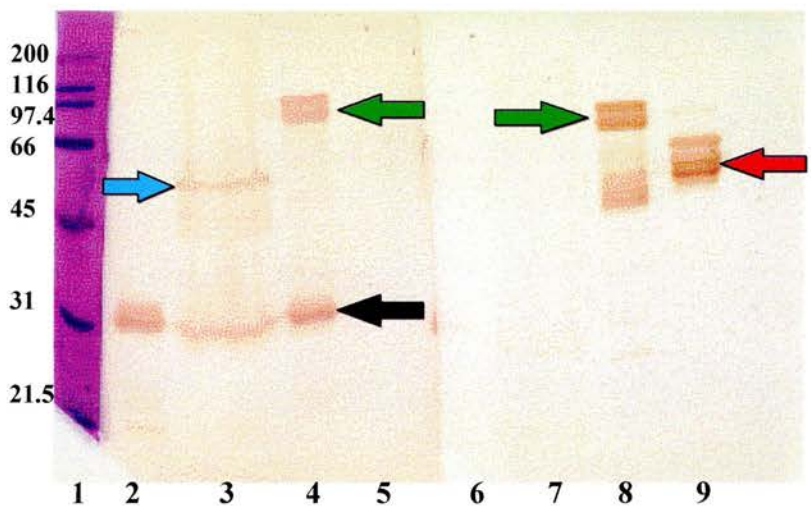

Figure 5.6 - Western Blot of gel identical to that shown in Figure 5.5 in which lanes 2-5 are repeated in lanes 6-9. Lane 1 comprises Coomassie brilliant blue (R-250) stained MW markers (kDa), lanes 2-5 probed with sheep anti-ENE 2A antibody and lanes 6-9 probed with sheep anti-equine API antibody. Purified ENE 2A in lanes 2 and 6 (black arrow); neutrophil extract (lanes 3 and 7); API:ENE 2A mixture (lanes 4 and 8); affinity purified equine API (red arrow, lanes 5 and 9). API:ENE 2A complex is detected with both anti-ENE 2A and anti-equine API antibodies (green arrows, lanes 4 & 8). The 55kDa band in the neutrophil extract (blue arrow, lane 3) may represent ENE 2A:HLEI complex.

Two bands of proteolysis of lower MW than uncomplexed ENE 2A were also detected demonstrating the presence of other proteases in the neutrophil extract.

Purified API demonstrates no proteolytic activity but the API:ENE 2A complex shows a trace amount (Figure 5.7).

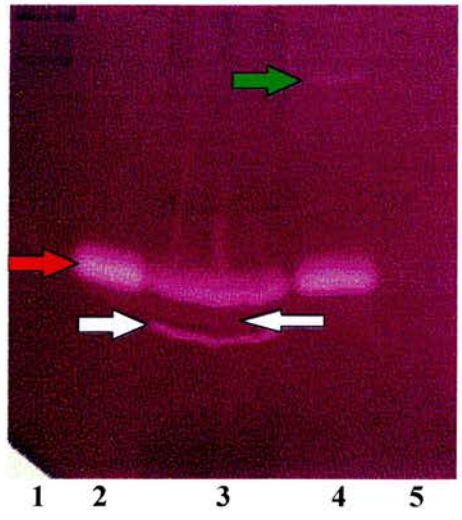

Figure 5.7 - Coomassie brilliant blue (R-250) stained 10% SDS-PAGE under non-reducing conditions with azocasein incorporated into the resolving gel to show proteolysis. The loading is identical to that described for the gel in Figure 5.5. Note proteolytic activity (denoted by clear bands) in lane 2 corresponding to purified ENE 2A (red arrow) and also at the same MW in lanes 3 and 4 (neutrophil extract and API:ENE 2A mixture respectively). Affinity purified API shows no proteolytic activity as expected (lane 5). API:ENE 2A complex show a small amount of proteolytic activity (green arrow, lane 4). Two areas of proteolytic activity are seen at lower MW than ENE 2A in the neutrophil extract (white arrows, lane 3).

5.3.4 Immunolocalisation of ENE 2A in Peripheral Blood Leukocytes

Immunocytochemistry of a mixed population of equine peripheral blood leukocytes with the sheep anti-ENE 2A antibody clearly demonstrates the presence of ENE 2A in multiple cytoplasmic granules confined to neutrophils (Figure 5.8). All cell nuclei were negative for ENE 2A (Figure 5.8). Negative control preparations were devoid of immunostaining (Figure 5.9).

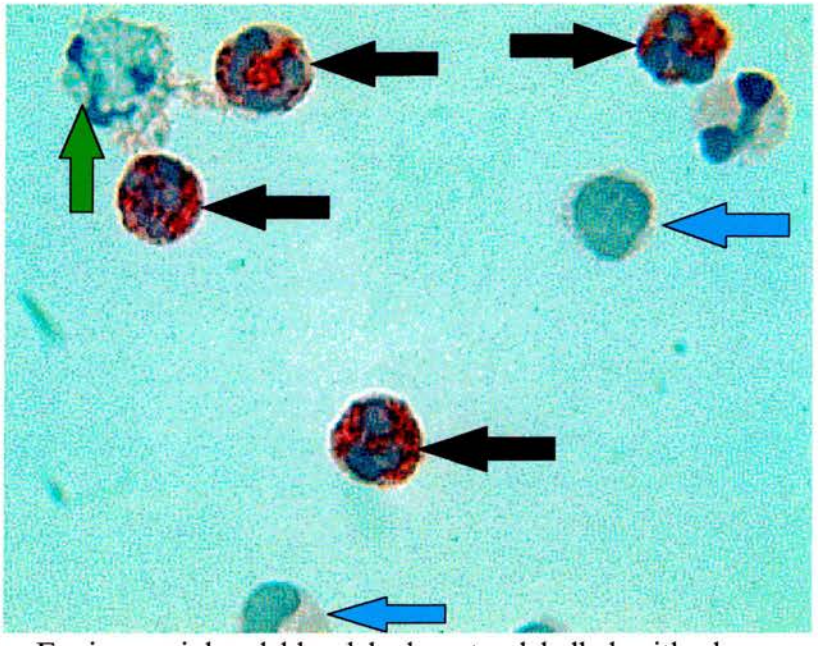

Figure 5.8 - Equine peripheral blood leukocytes labelled with sheep anti-ENE 2A antibody, rabbit anti-sheep IgG:HRPO with AEC substrate (red pigment) showing location of ENE 2A. Neutrophils (black arrows) are easily identifiable by their multilobed nuclei and the abundant red granular staining cytoplasm. Eosinophils (green arrow) and monocytes (blue arrows) are devoid of any immunostaining for ENE 2A (x 1000).

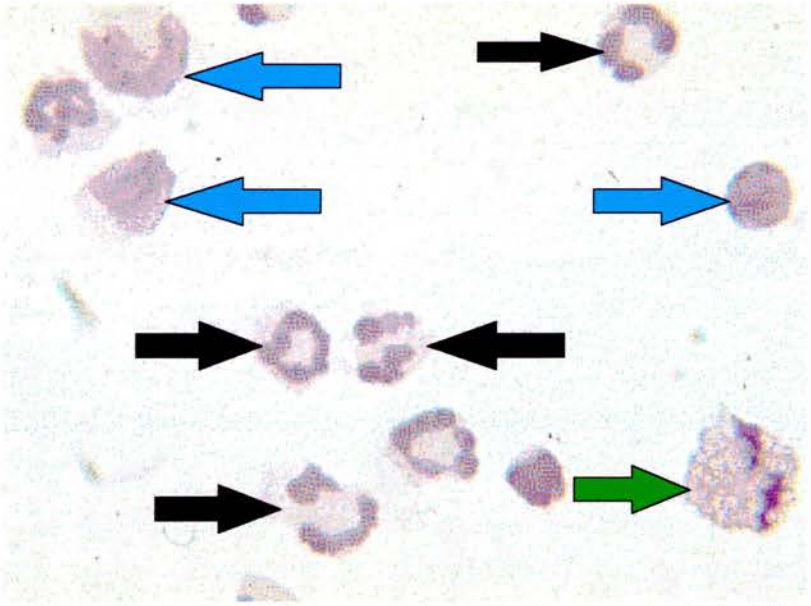

Figure 5.9 - For control purposes normal sheep IgG was substituted for sheep anti-ENE 2A antibody. The leukocytes (neutrophils denoted by black arrows, eosinophil by green arrows and monocytes by blue arrows) are devoid of any immunostaining for ENE 2A (x 1000).

5.3.5 Evaluation of ENE 2A and API in Peripheral Blood Leukocytes by Dual Immunofluorescence

Immunofluorescence of a mixed population of peripheral blood leukocytes with sheep anti-ENE antibody clearly demonstrated the presence of ENE 2A in multiple cytosolic granules in neutrophils (Figure 5.10). Other cell types were negative for ENE 2A as were all cell nuclei. In contrast a more uniform, less intense and non-granular pattern of immunostaining was seen in the cytosol of neutrophils when probed with the sheep anti-equine API antibody (Figure 5.11). Cytoplasmic staining of equine API is also present in some mononuclear leukocytes. Double immunofluorescence emphasises the contrasting patterns of distribution of API and ENE 2A in the cytosol of neutrophils. However, the two proteins do appear to patially colocalize within the cytoplasm of the neutrophils at some points (Figure 5.12). Negative control preparations were devoid of immunofluorescence.

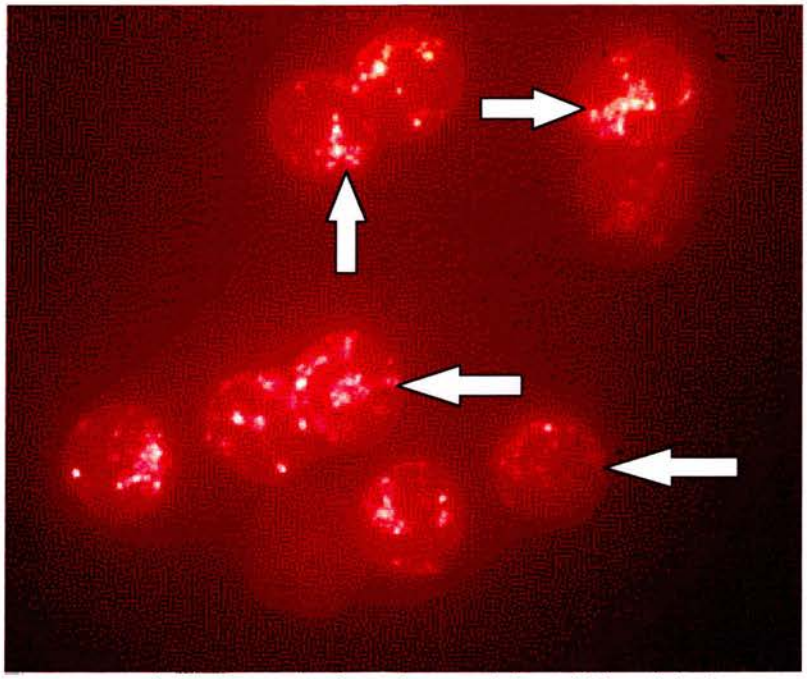

Figure 5.10 - Immunofluorescence of equine peripheral blood leukocytes with sheep anti-ENE 2A:Cy3 confirms that ENE 2A is confined to neutrophil granules (white arrows, x 1000).

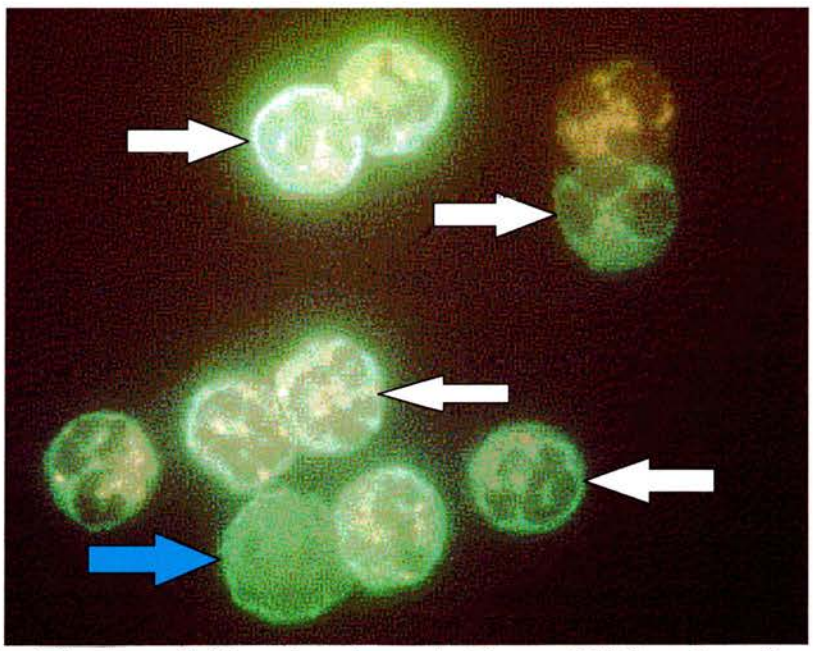

Figure 5.11 - The same field as that shown in Figure 5.10 but the cells are detected with anti-equine API:FITC labelled antibody. The cytoplasm of neutrophils is diffusely and variably immunofluorescent for equine API (white arrows). The cytoplasm of a mononuclear cell (blue arrow) also immunofluoresces for equine API (x 1000).

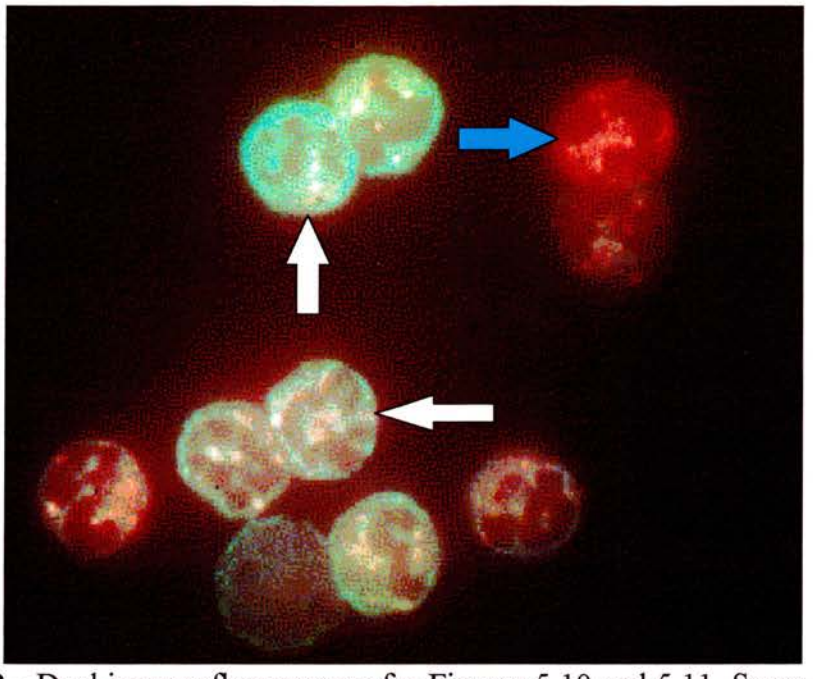

Figure 5.12 - Dual immunofluorescence for Figures 5.10 and 5.11. Some neutrophils show little equine API immunofluorescence (blue arrow). There is some degree of colocalisation between ENE 2A and API in neutrophils that immunofluoresce for both proteins (x 1000).

5.3.6 Evaluation of the Repeatability of the ENE 2A Specific Sandwich ELISA

Intra and inter-plate variations in quantification of ENE 2A by the polyclonal sandwich ELISA as represented by coefficients of variance (described in 5.2.7) were 4.3% (n = 64) and 16.1% (n = 18) respectively.

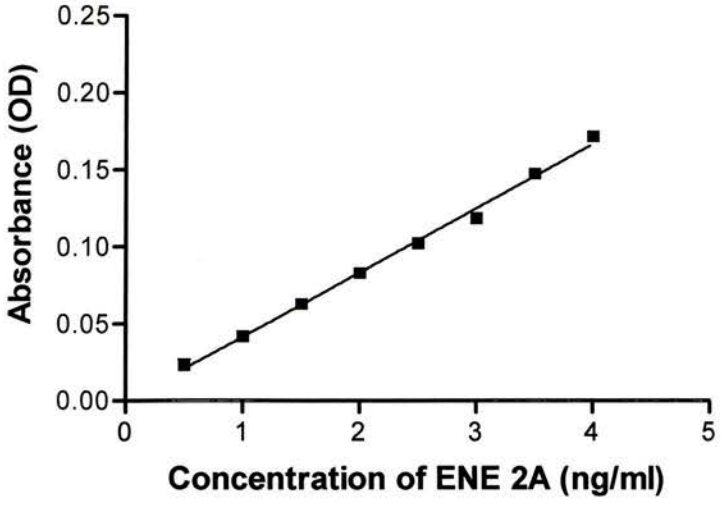

Figure 5.13 - Graph of absorbance (Optical Density) versus concentration of ENE 2A for the standard curve (mean ± maximum and minimum of 3 values) in the specific ELISA to measure the latter. The curve was used to derive ENE 2A concentrations of diluted samples whose optical densities gave ENE 2A values between 1-3.5ng/ml.

5.3.7 Evaluation of Sheep Anti-ENE 2A Antibody Recognition of Complexed ENE 2A by ELISA.

ENE 2A was recognised by ELISA whether in the free or complexed state. Analysis of the absorbance values by the Kruskal-Wallis test found no significant difference in OD values between free or complexed ENE 2A ($p = 0.07$).

5.3.8 Determination of the Amount of ENE 2A and Equine API in a Peripheral Blood Neutrophil

The specific polyclonal sandwich ELISAs were used to quantify the neutrophil content of ENE 2A and equine API independent of any enzymatic or inhibitory properties. The values per neutrophil were 0.813pg ± 0.179 and 0.021pg ± 0.011 (mean ± SEM) for ENE 2A and API respectively.

5.4 Discussion

The original protein purification procedure (Dubin *et al* 1976) based on ion-exchange chromatography resulted in relatively pure ENE 2A but with detectable high and low MW impurities when subjected to silver stained SDS-PAGE (Figure 5.1) which were easily removed by MW exclusion chromatography (Figure 5.2). The resultant ENE 2A (≈100% pure, Figure 5.3) was used to raise specific antibodies and as a ligand both for affinity purification of specific antibody and as a standard curve for a sandwich ELISA to measure ENE 2A. The MW of the purified ENE 2A (31kDa) as determined by SDS-PAGE under reducing conditions is in agreement with that of a recent study (Scudamore *et al* 1993).

The sheep anti-ENE 2A antibody is highly specific and recognises its antigen (ENE 2A) on Western blots and ELISA regardless of whether it is in the free or complexed state (Figure 5.6). Monoclonal antibody 1B also recognises its antigen (equine API) in the free and complexed state when used to probe Western blots of immobilised antigens, the combined results from the two antibodies can be used to identify ENE 2A:API complexes (Figure 5.6). The ability to recognise ENE 2A in the free or complexed state to a similar degree is advantageous as this will result in measurement of total ENE 2A by the ELISA in biological samples.

The presence of two separate bands of proteolysis on the azocasein gel (Figure 5.7), both of lower MW than ENE 2A, suggests the presence of other proteolytic enzymes in the neutrophil extract. ENE 2B is reported as having a lower MW than ENE 2A as

well as a lower concentration per neutrophil (Dubin and Koj, 1986). One of these bands denoting proteolysis may represent ENE 2B emphasising how immunologically distinct from ENE 2A it is as no corresponding band is seen on the Western blot of an identical gel probed with the sheep anti-ENE 2A antibody (Figure 5.6). The identity of the third band of proteolysis found on the azocasein gel is unknown as the only other proteolytic enzyme described in equine neutrophils is proteinase 1 which was shown to have a higher molecular weight than ENE 2A (Dubin *et al* 1976; Potempa, 1982). No other proteolytic enzymes have been isolated from equine neutrophils.

Equine neutrophils have been reported to contain about one third the neutrophil elastase found in human neutrophils (Dubin and Koj, 1986; Campbell *et al* 1989) and this has been proposed as a possible explanation for the rarity of emphysema in the horse (Edelman *et al* 1992). Using a specific sandwich ELISA we have shown the amount of ENE 2A per neutrophil to be over 0.8 pg/cell which is 100% greater than previous estimates derived by evaluation of enzyme activity (Dubin and Koj, 1986). In addition, equine neutrophils contain the immunologically distinct elastase, ENE 2B, at approximately a third the concentration and 5 times the elastinolytic activity of ENE 2A (Dubin *et al* 1976) suggesting that the total amount of ENE per equine neutrophil is even closer to the figure accepted for human neutrophils (1.1pg/cell) (Campbell *et al* 1989). The presence of HLEI (Dubin, 1977; Potempa *et al* 1988; Dubin *et al* 1992; Kordula *et al* 1993) and API in equine neutrophils implies that measurement of ENE by an immunological method, independent of enzyme activity, may be a more accurate means of determining total elastase concentrations. On Western blots, the presence of

a 55kDa band in horse neutrophil extract (Figure 5.6) which was detected with the sheep anti-ENE 2A antibody but has no demonstrable proteolytic activity confirms the presence of ENE 2A complexes (Figure 5.7). Human neutrophils are known to contain endogenous inhibitors, notably API and secretory leukocyte proteinase inhibitor (SLPI), the latter is more abundant and therefore probably the most important cytosolic elastase inhibitor (duBois *et al* 1991; Sallenave *et al* 1997a). This study has confirmed the presence of API in equine neutrophils at a low concentration (0.02pg/cell) indicating that it may similarly have a minor role to play as HLEI is reported to be present at 0.5pg/cell (Dubin and Koj, 1986). The different distributions of API and elastase in horse neutrophils also suggests that they do not completely colocalise and therefore elastase is unlikely to be fully regulated by API. This is supported by the lack of immunoreactive API:ENE 2A complexes seen on Western blots of crude neutrophil extracts despite the presence of the 55kDa band suggesting this is a complex of ENE 2A (31kDa) and HLEI (35.2kDa) and not ENE 2A and API which would have a molecular weight from 79-108kDa (Figure 5.6).

These results, contrary to the accepted figures, show that horses and humans have similar concentrations of neutrophil elastase per peripheral blood neutrophil. In terms of their respective elastinolytic activity, ENE 2B is reported to have seven times that of HNE which has similar elastinolytic activity to that ENE 2A (Dubin and Koj, 1986), again suggesting that the overall elastinolytic potential between the two species is similar.

The ELISA developed in this study will be of great value in determining the relative amounts of ENE 2A released from Percoll purified equine peripheral blood neutrophils stimulated with secretagogues under various conditions.

Chapter 6

KINETICS OF EQUINE NEUTROPHIL ELASTASE 2A RELEASE AND SUPEROXIDE ANION GENERATION FOLLOWING ACTIVATION BY ZYMOSAN ACTIVATED SERUM

6.1 Introduction

Equine neutrophils have been shown to significantly differ in their functional responses to various stimuli in comparison to neutrophils from other species (Styrt, 1989). Reduced release of superoxide anion radicals (SOA) is seen in equine neutrophils stimulated with N-formyl-methionyl-leucyl-phenylalanine (fMLP) compared to humans neutrophils (Brazil *et al* 1998). This is thought to be due to equine neutrophils having fewer receptors with a single affinity for fMLP (Snyderman and Pike, 1980) compared to human neutrophils which have more cell surface fMLP receptors and of two differing affinities (Snyderman and Goetzl, 1981; Snyderman and Pike, 1984; Snyderman, 1984). Furthermore, equine neutrophils, in contrast to human neutrophils, equine eosinophils and porcine granulocytes, are unable to produce leukotrienes when stimulated solely with the calcium ionophore A23187 (Lindberg *et al* 1998). However, addition of exogenous arachidonic acid along with ionophore to purified equine peripheral blood neutrophils results in the production of leukotriene B_4 suggesting that elevation of intracellular calcium is insufficient in itself

to induce utilisation of endogenous arachidonic acid from cell membranes in horse neutrophils (Lindberg *et al* 1998).

The most consistent chemotactic response across the species appears to be to activated homologous plasma or serum (reviewed in Styrt, 1989). The complement fragment C5a is presumed to be the major active component in activated serum or plasma, but other factors or complement fragments may also play a role. This suggests that there is a receptor for complement fragments on neutrophils of all the mammalian species so far studied; human, non-human primate, equine, bovine, feline, rabbit, guinea pig, mouse and rat (reviewed in Styrt, 1989).

There are however marked species differences in response to other chemotaxins. For example equine neutrophils have been shown to be unresponsive to equine API:ENE 2A complex (Scudamore *et al* 1993), the human equivalent being highly chemotactic for human neutrophils (Banda *et al* 1988). Neutrophil chemotaxis to fMLP, which has been well characterised, is also species dependent; primate, rat, rabbit and guinea pig neutrophils all respond chemotactically to fMLP whereas porcine, feline, canine and equine neutrophils do not (reviewed in Styrt, 1989). However, fMLP can induce equine neutrophils to secrete lysosomal enzymes (Camp and Leid, 1982; Sedgwick *et al* 1987; Fittschen *et al* 1988; Mcewen and Lumsden, 1991). The presence of a single type of fMLP receptor on the equine neutrophil (Snyderman and Pike, 1980) has again been proposed as a possible explanation for this difference as stimulation of

different fMLP receptors on human neutrophils may induce different cellular responses (Styrt, 1989).

Human neutrophils have been shown to release HNE and SOA concurrently when stimulated with either fMLP, phorbol-myristate-acetate (PMA) or the calcium ionophore A23187 (Khalfi *et al* 1998). Interestingly, opsonised zymosan (OZ), which induces a respiratory burst, does not induce human neutrophils to degranulate and release HNE (Khalfi *et al* 1998) suggesting a different stimulatory mechanism or pathway compared to fMLP, PMA and A23187. Inhibition of protein kinase C (PKC) prior to stimulation of human neutrophils with PMA, dioctanoylglycerol, fMLP, calcium ionophore A23187 or OZ significantly abrogates the production of SOA suggesting this enzyme is critical to the process (Cabanis *et al* 1996). However, inhibition of PKC only appears to abrogate PMA mediated elastase release, not that induced by fMLP and A23187 (Cabanis *et al* 1996). This suggests that fMLP and A23187 do not mediate elastase release through PKC despite producing a similar SOA response compared to PMA. Contrary to the human scenario (Khalfi *et al* 1998), buffalo neutrophils release NE when stimulated with OZ (Singh *et al* 1997) illustrating further species variations in the functional responses to identical stimuli.

Unregulated and/or excessive elastase activity has been shown to be responsible for pulmonary emphysema in all species so far studied (Laurell and Eriksson, 1963; Hayes *et al* 1975; Janoff *et al* 1977; Janoff *et al* 1979; Janoff, 1985a) yet specific measurement of NE released from stimulated equine neutrophils has not been

previously documented. The main inhibitor of NE in the lower respiratory tract of humans is API (Gadek *et al* 1981a) which has been shown to be oxidatively inactivated *in vitro* by SOA from stimulated human neutrophils (Matheson *et al* 1981; Ras *et al* 1992). Inactivation of API could lead to an increase in half-life of free NE secreted into the microenvironment around activated neutrophils (Johnson and Travis, 1979; Matheson *et al* 1982). For this proposed synergism to occur there must be some temporal relationship between the release of these two neutrophil products.

Species differences in functional responses to various stimuli highlight the need to study neutrophils specifically from the species of interest and to extrapolate with caution from the data for other species. Differences between equine and human neutrophils with respect to SOA generation and release of NE in terms of response to various stimuli and temporal co-ordination of these responses may affect the relative proteinase:antiproteinase balance resulting in species variations in susceptibility and development of pulmonary emphysema.

The aims of this study were to investigate the characteristics of ENE 2A release and of SOA generation from equine peripheral blood neutrophils stimulated with zymosan activated serum (ZAS). The dependence of these processes on extracellular divalent cations was also investigated in an attempt to elucidate the mechanism through which the ZAS stimulus was mediated.

6.2 Materials and Methods

6.2.1 Preparation of Zymosan Activated Serum

Zymosan activated serum was used as a source of the complement fragment C5a. Serum was chosen as opposed to plasma as clotting of the latter interfered with the sampling procedure for evaluation of ENE 2A release from stimulated neutrophils (see below). Serum was prepared by restoring calcium to platelet rich equine plasma (a by-product of the neutrophil purification procedure, see Chapter 5.2.2) by addition of 220µl of 10mM CaCl/10ml followed by incubation for 90 minutes at 37°C in an humidified 5% CO_2 atmosphere. Zymosan was mixed with the serum (5mg/ml) by vortexing and sonication. When all the zymosan was suspended, the mixture was incubated at 37°C for 60 minutes followed by centrifugation at 1400 x g for 15 minutes and by separation of the supernatant from the insoluble zymosan pellet. Heat inactivated zymosan activated serum (HIZAS) was produced by an identical procedure except that the serum was heat inactivated at 56°C for 30 minutes before addition of zymosan. Both supernatants were stored in aliquots at -70°C.

6.2.2 ZAS Stimulation of SOA Generation

A sample of the Percoll purified peripheral blood neutrophils, isolated as described in Chapter 5.2.2, was resuspended at 12.5 x 10^6 cells/ml in Dulbecco's PBS/0.1% BSA at 37°C. Detection of extracellular SOA was by lucigenin-dependent chemiluminescence (LDCL) (Allen, 1986; Benbarek *et al* 1996) using a ML 3000 microtitre plate luminometer (Dynex Technologies, Billingshurst, West Sussex, UK). 100µl lucigenin (0.25mM in Dulbecco's PBS/0.1% BSA at 37°C) and 1 x 10^6 freshly

isolated cells (80µl) were added to each well. After a 5 minute equilibration phase, basal LDCL was recorded over 12 minutes to ensure that cells were not basally activated. A dose response curve was derived for SOA generation in response to ZAS stimulation; 20µl of either undiluted ZAS, or doubling dilutions of ZAS with Dulbecco's PBS/0.1% BSA (1/2, 1/4 and 1/8), were used to stimulate SOA release from Percoll purified equine peripheral blood neutrophils (5 x 10^6 cells/ml final concentration), samples were performed in triplicate (n = 1 individual horse). After determination of optimal stimulation, experiments were performed by addition of 20µl of buffer (PBS), undiluted ZAS, HIZAS or ZAS + EGTA (final conc. EGTA 4mM). Basal and agonist stimulated chemiluminescence were recorded to produce mean peak and integral (area under the curve) chemiluminescence values over a 90 minute period for 6 individual horses.

6.2.3 ZAS Stimulation of ENE 2A Release

The remaining purified neutrophils were resuspended at 5.56 x 10^6 cells/ml in Dulbecco's PBS /0.1% BSA and 4.5ml aliquots placed in universal containers. A dose response curve was derived for release of ENE 2A from Percoll purified peripheral blood neutrophils in response to ZAS stimulation. Undiluted ZAS (0.5ml) or doubling dilutions of ZAS with Dulbecco's PBS/0.1% BSA (1/2, 1/4 and 1/8) were added to stimulate ENE 2A release from the neutrophils and the concentrations measured by the ELISA described in chapter 5.2.7 (n = 1 individual horse). After determination of the stimulation required for optimal release of ENE 2A, experiments were performed by addition of 0.5ml of either PBS, undiluted ZAS, HIZAS or ZAS with EGTA (final

conc. EGTA 4mM) to separate identical universal containers, into which 4.5ml of purified neutrophils had already been placed. This gave a final cell concentration of 5 x 10^6 cells/ml and was followed by gentle mixing. Samples (0.5ml) were withdrawn at 0, 15, 30, 45, 60, 90, 120 and 150 minutes. Each sample was centrifuged at 13800 x g for 10 minutes at 4°C, the supernatant was decanted and stored at -70°C until analysis by ELISA for ENE 2A concentration as described in chapter 5.2.7 (n = 6 individual horses).

6.2.4 Cell Viability

Cell viability was assessed by trypan blue exclusion (Chapter 2.14) at time zero and 150 minutes post stimulation for samples exposed to each of the four stimuli.

6.3 Results

6.3.1 Dose Response Curves

Superoxide Anion Generation. Undiluted ZAS produced the maximum generation of SOA as detected by LDCL with a peak at 35 minutes post stimulation and abrogation of the response by 90 minutes. The response was markedly attenuated by a two fold dilution of ZAS and further reduced by four and eight fold dilutions (Figure 6.1). PBS produced no change from the basal state.

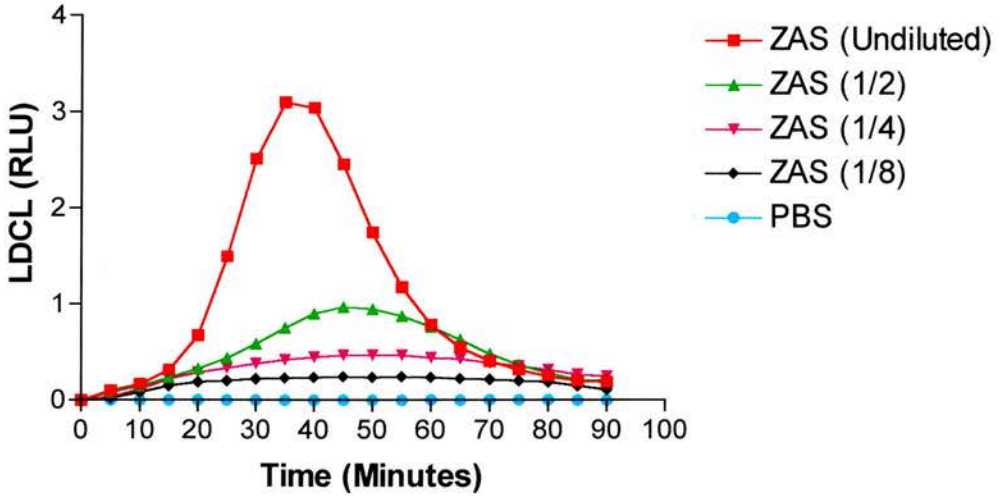

Figure 6.1 - Dose response curve of SOA generation from Percoll purified equine peripheral blood neutrophils with doubling dilutions of ZAS as detected by lucigenin dependent chemiluminescence (LDCL) measured in relative light units (RLU). Values are mean of triplicate wells. Note maximal generation of SOA by undiluted ZAS.

ENE 2A Release in Response to ZAS. Undiluted ZAS stimulated the maximal release of ENE 2A with the dilutions of ZAS (1/2, 1/4 and 1/8) resulting in progressively less ENE 2A being released (Figure 6.2). Increases were noticeable at 60 minutes but much greater by 120 minutes. After 60 minutes neutrophils treated with PBS show some 'leakage' of ENE 2A which could be due to suboptimal media for maintaining cells *in vitro*, particularly the lack of serum components.

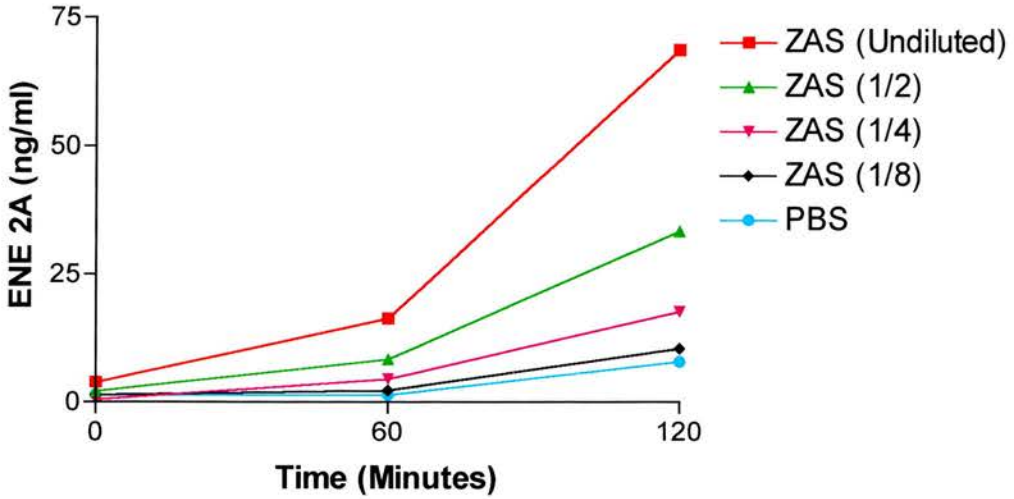

Figure 6.2 - Dose response curve of ENE 2A release from Percoll purified equine peripheral blood neutrophils with doubling dilutions of ZAS as detected by ELISA. Note the undiluted ZAS gave the maximal release of ENE 2A.

6.3.2 Concurrent Generation of SOA and Release of ENE 2A From ZAS Stimulated Equine Peripheral blood Neutrophils

Undiluted ZAS produced the largest generation of SOA and release of ENE 2A from Percoll purified equine peripheral blood neutrophils and was used as a stimulus in all subsequent experiments. The ZAS induced respiratory burst produced a peak SOA generation at 25 minutes post stimulation with complete desensitisation of the response by 90 minutes despite the continued presence of the agonist (Figure 6.3). Comparison of the total amount of SOA produced over 90 minutes, as represented by the areas under the curves, show that a statistically significant greater quantity of SOA was generated by neutrophils stimulated with ZAS when compared (Kruskal-Wallis test) to the amounts generated in response to the other three stimuli (ZAS vs. HIZAS $p = 0.01$, ZAS vs. PBS $p = 0.005$ and ZAS vs. ZAS + EGTA $p = 0.005$). Stimulation with HIZAS resulted in similar kinetics for SOA generation but with a significant reduction in magnitude (ZAS vs. HIZAS $p = 0.01$). Cells stimulated with

PBS or ZAS + EGTA released negligible levels of SOA. Zymosan activated serum stimulation in the presence of EGTA abolished the SOA generation such that the LDCL response was not significantly different to control cells treated with PBS (p = 0.9).

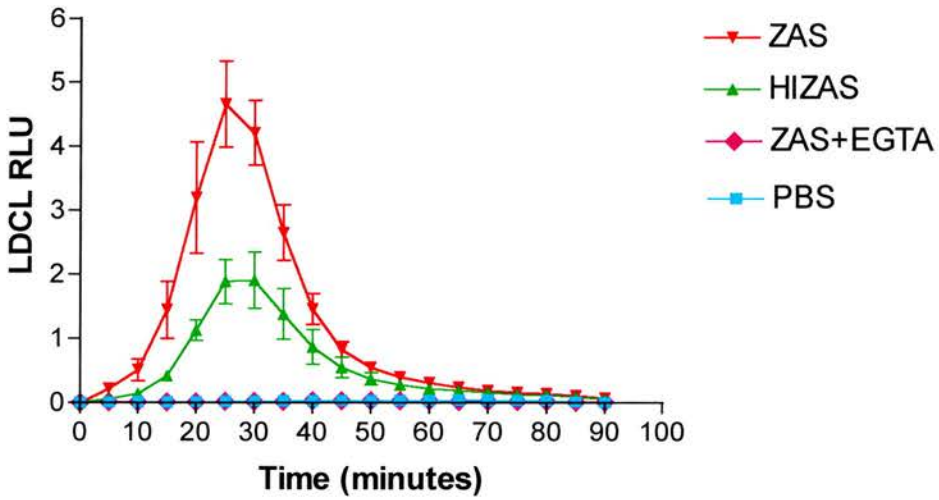

Figure 6.3 - SOA generation from ZAS, HIZAS, ZAS+EGTA and PBS stimulated equine neutrophils detected by LDCL (relative light units, RLU, mean ± SEM of 6 individual horses) over 90 minutes. Note peak of SOA generation in both ZAS and HIZAS is at 25 minutes post stimulation and total abrogation of response occurs by 90 minutes.

In contrast, the kinetics of ENE 2A release (in response to ZAS stimulation) were prolonged with continuing accumulation in supernatants for up to 150 minutes (the final sampling point, Figure 6.4). ZAS stimulated neutrophils consistently released significantly greater amounts of ENE 2A over the whole 150 minutes (when analysed by Kruskal-Wallis test) compared with values for PBS (p = 0.005). However, the ENE 2A released after stimulation with ZAS is significantly greater than that generated in the presence of HIZAS over the first 90 minutes (p = 0.03-0.005). Interestingly, EGTA did not completely abrogate ENE 2A release in response to ZAS

as there is no statistical difference from ZAS treated cells in the amount of ENE 2A at time zero ($p = 0.1$). The amount of ENE 2A released from neutrophils stimulated with ZAS + EGTA varies very little from the initial value at time zero suggesting that there is little or no release of ENE 2A after this. Over the initial 90 minutes there is a significant difference between the amount of ENE 2A released from neutrophils treated with ZAS + EGTA and those treated with PBS ($p = 0.03$-0.005). By 120 minutes the 'leakage' of ENE 2A from cells treated with PBS has risen to a level whereby the difference between the two stimuli is no longer significant ($p = 0.07$).

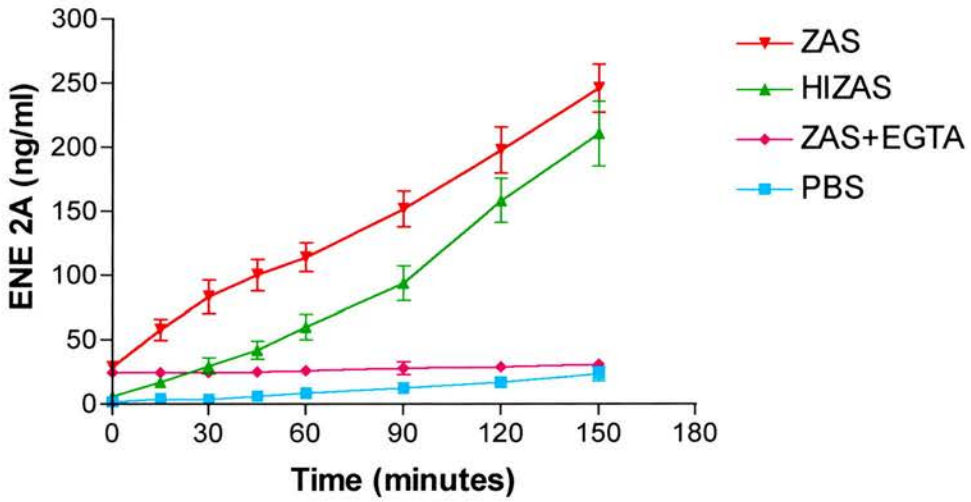

Figure 6.4 - Cumulative release of ENE 2A from Percoll purified equine peripheral blood neutrophils stimulated with ZAS, HIZAS, ZAS+EGTA and PBS, mean ± SEM of 6 individual horses. Note different characteristics of release pattern compared to SOA generation (Figure 6.3) and the consistently higher concentration of ENE 2A in the ZAS + EGTA treated neutrophils compared to those treated with PBS.

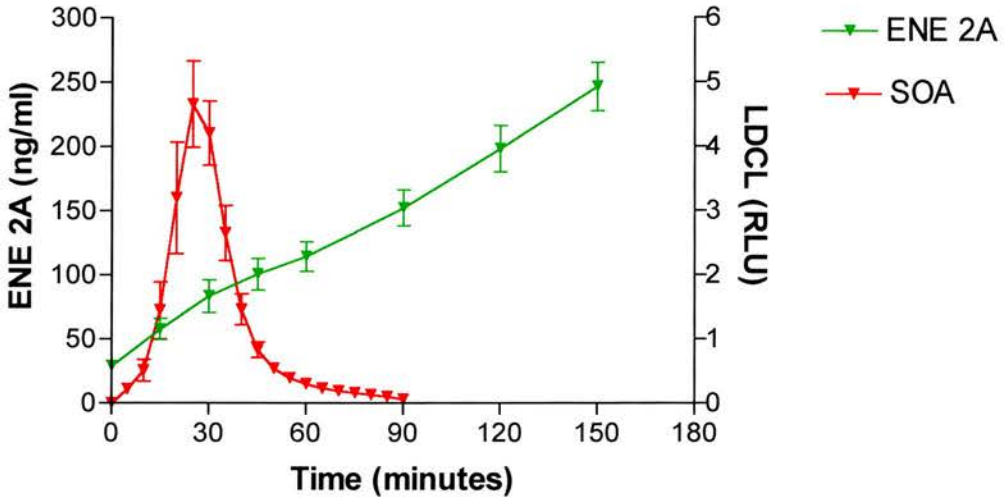

Figure 6.5 - Comparison of the levels of SOA and ENE 2A over 150 minutes post stimulation of equine neutrophils by ZAS. Note rapid burst of SOA compared to slower and more prolonged release of ENE 2A.

6.3.3 Cell Viability

Cell viability, as denoted by trypan blue exclusion, was 100% (n = 6 for each of the four stimuli) at time zero in all four stimuli. At 150 minutes post stimulus mean cell viability in all cases was still > 99%.

6.4 Discussion

The presence of complement fragment receptors on the surface of neutrophils from previous chemotactic studies in all mammalian species so far examined (Styrt, 1989), including horses (Scudamore *et al* 1993) suggested that activated complement plays a major role in neutrophil stimulation *in vivo*. This prompted the choice of ZAS in this study to evaluate the effect of a single stimulus on concurrent SOA generation and release of ENE 2A from equine neutrophils.

The relationship between total SOA generation and total ENE 2A release during the initial 90 minutes post ZAS stimulation (the duration of the oxidative burst) suggests that the extra-cellular release of the two products are closely linked, however, the kinetics of release are markedly different. Superoxide anions are generated and released in a respiratory burst lasting about 90 minutes with a peak at 25 minutes. In contrast elastase release is initiated at the start of the oxidative burst and then continues to accumulate at a steady rate leading to a nearly linear increase in the extracellular concentration for at least 150 minutes post ZAS stimulation. Human API and equine Spi1 are oxidation sensitive due to the presence of methionine residues at critical positions in the API molecules. The early peak of superoxide anion release may act to down regulate serpin inhibition of elastase activity in the extracellular micro-environment of the neutrophil by reducing API's K_{ass} and K_i with neutrophil elastase (Cohen, 1979; Nakajima *et al* 1979; Beatty *et al* 1980; Travis *et al* 1980; Hubbard *et al* 1987; Potempa *et al* 1991b; Snider, 1992; Buhl *et al* 1996).

These findings indicate that, while there may be a common signalling pathway initiating superoxide anion and elastase release (Roos, 1991), the post initiation mechanisms of release are clearly different. Superoxide anion is known to be generated by NADPH oxidase which can become refractory despite continued presence of the stimulus (Rossi, 1986; Roos, 1991). However, elastase release does not appear to be refractory to continued presence of the stimulus throughout the time course of these experiments. The continued presence of the stimulus may mimic the *in vivo* situation where inflammatory stimuli and mediators may be present in the tissue for many minutes or hours. It is possible that this results in release of neutrophil elastase over a prolonged time period which with early superoxide anion assistance produces persistent low levels of proteolysis resulting in tissue damage. Interestingly, less than 10% of the total elastase content of equine neutrophils was released over the initial 90 minutes when the SOA respiratory burst is present suggesting that either not all of the ENE 2A present within equine peripheral blood neutrophils can be released or, that the majority is released after the respiratory burst. Synergy between SOA and ENE 2A would only be expected to occur in the initial 90 minutes post neutrophil stimulation when SOA are oxidatively inactivating API and for a short period of time afterwards until active API diffused into the area. This assumes that this *in vitro* situation represents what occurs *in vivo*. However, recent data (Mark Dagleish, preliminary study), has shown that the type of medium which the neutrophils are suspended in greatly affects the cells' SOA generating capacity. Media such as Iscove's modified Dulbecco's medium (IMDM) increases the amount of SOA generated and the time scale over which it is detected by LDCL compared to

Dulbecco's PBS when an identical secretagogue is used. This suggests that a more nutrient rich environment may enhance the amount and duration of the respiratory burst of stimulated neutrophils resulting in an increased time period of synergy between SOA generation and ENE 2A. The latter may be more representative of the *in vivo* situation where neutrophils would be bathed in extracellular tissue fluid.

It is intriguing that heat inactivation of ZAS does not completely remove its ability to stimulate neutrophil degranulation or SOA generation suggesting that there must be some stimulus present other than heat labile C5a. However, the heat labile components of ZAS do have a significant effect on the initial rate of degranulation and release of ENE 2A ($p = 0.005$) compared to HIZAS (Figure 6.4). Further studies are required to determine the exact nature of this heat resistant component.

Previous studies have shown SOA generation and HNE release from human neutrophils to be variably dependent on extracellular divalent cations, the concentration and the stimulus used (Khalfi *et al* 1996). SOA generation from human neutrophils is dependent on extracellular calcium when stimulated by fMLP but not when stimulated by PMA or OZ (Khalfi *et al* 1996). Elastase release from stimulated human neutrophils is more complicated. PMA stimulation is independent of calcium when the extracellular concentration ranges from zero to 1.0mM, but dose dependent from 1.5-4.0mM; greater concentrations of calcium resulting in more HNE release (Khalfi *et al* 1996). Conversely, fMLP induced HNE release is dependent upon

extracellular calcium from zero-1.5mM yet less affected at concentration greater than 1.5mM (Khalfi *et al* 1996).

In our study we found that the addition of EGTA abolishes ZAS stimulated superoxide anion generation suggesting that this response must be dependent on extracellular calcium and/or magnesium typical of a receptor mediated, secondary messenger signalling mechanism (Korchak *et al* 1984; Buhl *et al* 1994) and similar to fMLP stimulated human neutrophils (Khalfi *et al* 1996). However, ZAS-stimulated ENE 2A release from equine neutrophils does not appear to be entirely dependent upon extracellular calcium and/or magnesium. At the first time point the ENE 2A content of supernatant from ZAS + EGTA stimulated cells is the same as the initial ZAS stimulated value, which is significantly greater ($p = 0.005$) than that of the PBS control. This suggests a small degree of cation independence, utilisation of stored intracellular cations or a significant difference in the kinetics of ZAS stimulation compared to chelation of cations by EGTA to facilitate initial release. This phenomenon requires further study as human neutrophils have been shown not to require a cytosolic calcium transient for degranulation or oxidative burst when stimulated by some receptor mediated stimuli (IgG, Seetoo *et al* 1997).

The presence of significant levels of ENE 2A in some samples (ZAS and ZAS + EGTA) at time zero (Figure 6.4) is probably due to the time taken from addition of the stimulus to removal and storage of the supernatant after centrifugation at 4°C allowing some cellular response.

The absence of panacinar emphysema in horses with COPD (Kaup *et al* 1990), despite a pulmonary neutrophilia (Freeman *et al* 1993; Dixon *et al* 1995a), a comparable NE content to human neutrophils (Chapter 5) and concurrent temporally related release of SOA and ENE 2A in response to a physiological stimulus still needs to be addressed. Alpha-1-proteinase inhibitor is believed to be responsible for anti-elastase activity at the alveolar level (Hubbard *et al* 1989; Ohlsson, 1980). In contrast to human API, equine API contains oxidation resistant isoforms (Spi2, 3 & 4) which may be resistant to superoxide anion inactivation (Patterson and Bell, 1989; Potempa *et al* 1991b). Therefore the more abundant oxidation resistant Spi proteins in the horse may result in API with full inhibitory activity towards ENE 2A in the presence of SOA generation unlike the human scenario where all API is oxidation sensitive. Alternatively equine neutrophils, despite pulmonary sequestration, may not be stimulated to degranulate fully *in vivo* resulting in a reduced proteolytic burden in the pulmonary tissues.

Chapter 7

GENERAL DISCUSSION

Genetically depressed plasma API concentrations and their link with premature pulmonary emphysema in humans (Laurell and Eriksson, 1963) was the discovery that prompted extensive scientific research into this field of medicine. Despite large numbers of affected patients, the disease defied rational investigation after its initial discovery due to a poor understanding of the biochemistry and genetics of the condition. Thirty years later, human API deficiency can now be diagnosed prenatally (Cox, 1989) and potentially supplemented postnatally with purified proteins from the milk of sheep transfected with the human API gene (Wright *et al* 1991; Carver *et al* 1993) thereby ameliorating the otherwise inevitable progressive destruction of lung tissue.

Research into equine API was primarily initiated by the discovery that the horse was the only animal, besides man, to suffer from what was originally termed 'spontaneous pulmonary emphysema'. The horse was consequently proposed as a possible animal model for further investigation into human pulmonary emphysema (Gillespie and Tyler, 1969; Gerber, 1973). However, biochemical studies uncovered a host of differences between human and equine API (Patterson *et al* 1991; Potempa *et al* 1991b) that, along with species differences in the anatomical pattern of tissue destruction present in neutrophil associated emphysema (Kaup *et al* 1990; Creagh and Krausz, 1992), has since excluded the use of the horse as an animal model (reviewed in Chapter 1).

This study has investigated some aspects of equine API and its target proteinase ENE 2A in an attempt to establish if a difference in the equine proteinase:anti-proteinase balance may be responsible for the species difference in anatomical distribution of tissue destruction in pulmonary emphysema in humans and horses.

Detection of equine API in the liver, using immunohistochemistry, suggests that API is more abundant when compared to levels detected in normal human hepatocytes (Palmer *et al* 1974; Palmer and Wolfe, 1976; Ray *et al* 1977; Palmer *et al* 1977; Ray and Desmet, 1978). This may represent more abundant synthesis in the horse and could be the reason for the higher plasma concentration of equine API (3.5-4g/l, Patterson *et al* 1991) compared to that of humans (1-1.3g/l, Carrell, 1986). Interestingly, the mouse, with 3.5-5g/l of plasma API (Takahara and Sinohara, 1982), has a distribution of API in hepatocytes (Gauldie *et al* 1980) which is similar to that described in the horse. This suggests that hepatic immunolocalisation may purely reflect the level of hepatic synthesis and consequent plasma levels of API. However, in rats, which have a plasma API concentration close to that of humans (1.7g/l, Koj *et al* 1978), hepatocytes are all positive for API in normal livers (Lamri *et al* 1986).

An alternative explanation is that there are methodological differences in the various studies. This is supported by the findings of Feldmann et al, 1976, where, like the present and the rat liver studies (Lamri *et al* 1986), paraformaldehyde was used as a fixative. This was the only record of API being detected by immunohistochemical

means in hepatocytes from normal PiMM human liver biopsies. All of these data support the theory that methodology is a key factor in successful immunodetection of API in normal hepatocytes. The immunohistochemical detection of API in human, mouse and rat tissues did not involve an antigen recovery procedure, as in the present study, suggesting that this may be responsible, in part, for the even more extensive staining of API in equine liver tissue.

Direct evidence of hepatic synthesis of equine API has not yet been shown. However, the diffuse staining of equine API in the cytoplasm of hepatocytes and the granular inclusions, which were often found in the perinuclear region (Chapter 4.3.2), are highly suggestive of synthesis in the endoplasmic reticulum followed by post translational modification in the Golgi complex prior to secretion. Intense staining of equine API in the bile ductules is consistent with the highly conserved C-terminal peptide of the API molecule being hydrophobic which is thought to be responsible for its association with various bile salts in humans, the precise role of this association is still under investigation (Eriksson, 1996).

With the exception of the liver, no significant disparity in the distribution or the cellular content of API was found between human and equine tissues. This would suggest a similarity of tissue functions for API in both species despite the different biochemical properties of equine and human API.

Earlier studies suggested that the elastase content of an equine neutrophil was considered to be about a third (Dubin and Koj, 1986) that of a human neutrophil (Tetley *et al* 1989) and consisting mainly of a type that has similar elastinolytic activity to HNE (Dubin and Koj, 1986). However, this study has emphasised the importance of methodology when investigating enzymes and their inhibitors, especially if there is any degree of colocalisation of the two in the tissues from which they are purified. With the aid of immunological techniques it has now been shown that the equine neutrophil contains over twice as much ENE 2A as previously calculated. The lack of analysis of ENE 2B, independent of enzymatic activity, is an important piece of the overall picture which needs to be addressed. Until this data, along with those of release characteristics, are available a true comparison of elastinolytic potential between equine and human neutrophils cannot be made.

At present all that can be surmised is that there is far more elastase present in an equine neutrophil than was originally thought. The published relative elastinolytic activity of ENE 2B being seven times greater than HNE, which in turn has similar elastinolytic activity to ENE 2A need to be re-evaluated after careful purification and kinetic studies using modern techniques.

Evidence of oxidative inactivation of human API is extensive (Cohen, 1979; Johnson and Travis, 1979; Travis *et al* 1980; Beatty *et al* 1980; Matheson *et al* 1982; Beatty *et al* 1984; Hubbard *et al* 1987). However, some question its occurrence *in vivo* (Campbell *et al* 1982) suggesting that API is excluded from the immediate micro-

environment, the neutrophil membrane forming a protective enclave for the released HNE and SOA. Despite studies showing actual oxidative inactivation of API by SOA from activated neutrophils (Ossanna *et al* 1986; Padrines *et al* 1989; Ottonello *et al* 1992; Ras *et al* 1992) and alveolar macrophages (Hubbard *et al* 1987) *in vitro* there has been little published data on the kinetics of concurrent extracellular detection of SOA and NE from neutrophils stimulated with physiological stimuli. Equine neutrophils have been shown in this study to generate and release SOA and secrete ENE 2A into the extracellular milieu in a correlated manner that would suggest the possibility of synergy and the potential for host tissue damage. Synergy is likely in the initial 90 minutes post stimulation with ZAS *in vitro*, whereas, *in vivo,* this may occur over a longer period due to the possibility of greater and prolonged SOA generation when cells are bathed in tissue fluid.

API regulates and protects against the barrage of SOAs and against ENE 2A in the lower respiratory tract in the horse. Not only is the concentration of equine serum API (Patterson *et al* 1991), and consequently by diffusion the concentration of pulmonary API, greater than that of humans (Carrell, 1986), but some equine API isoforms are resistant to oxidative inactivation (Patterson *et al* 1991). The majority of the oxidation resistant APIs (Spi3A and B) have a K_{ass} at least two orders of magnitude less than that the oxidation sensitive Spi1 (Potempa *et al* 1991b; Alan Pemberton, unpublished data). This in itself could be critical as total abolition of extracellular NE activity would be disadvantageous to host defence. People suffering from Chediak-Higashi syndrome have defective neutrophils and are prone to

persistent bacterial infections (Dale *et al* 1972). It must be borne in mind that although we are investigating host tissue damage by neutrophils, they, and their microbicidal products are essential to host defence and ultimately survival.

The role of oxidation sensitive API as an inhibitor of SOA activity has not been investigated but it has the potential to play a significant role as an antioxidant for scavenging free radicals and thereby protecting host tissues. The importance of API as an antioxidant in pulmonary tissues needs to be considered more broadly rather than confining research purely to oxidative inactivation of API.

In conclusion this study, together with the previously published data, suggest that equine and human neutrophils contain comparable amounts of NE. Stimulation of neutrophils by ZAS results in extracellular SOA and ENE 2A which have the potential to act synergistically over the initial 90 minutes post-stimulation *in vitro* and probably longer *in vivo*. The differences in the degree and anatomical distribution of pulmonary emphysema in horses and humans is probably due to a more effective anti-elastase screen provided by a higher concentration of equine API in pulmonary epithelial lining fluid, derived from the plasma, combined with the presence of oxidation resistant Spi proteins resulting in more efficient inhibition of extracellular ENE in the neutrophil microenvironment. The horse, in having a plasma concentration of oxidation sensitive Spi1 comparable to that of humans, as well as oxidation resistant Spi proteins, has probably achieved a more efficient anti-elastase screen.

The evolutionary forces behind the horse developing a more efficient antiproteolytic screen are less than obvious. The wild horse originally inhabited several differing environments; tundra, moorland, marsh and forest etc. (Bennet, 1992), none of which would appear to subject the horse to greater proteolytic or pulmonary inflammatory inducing conditions than those experienced by early humans.

However, we may be looking at this the wrong way. What if there has been a selection pressure against humans having a high antiproteinase screen? Support for this theory comes from examination of the PiZ homozygous state in humans where the frequency of the Z gene is far too high to be present just by chance (Kueppers, 1972). This suggests there must have been some positive selection pressure for a less potent antiproteolytic screen. The most likely explanation for a higher gene prevalence would be if it affected fertility in a positive manner. One study found a proteinase in rabbit sperm that was inhibited by API and suggested that if it occurred in humans, sperm would be more able to penetrate the zona pellucida of ova in homo and heterozygotes for the PiZ allele resulting in greater fertility compared to PiMM individuals (Kueppers, 1972).

Another possible explanation for depressed API levels resulting in increased fertility in PiZ homo and heterozygotes could be species variations in placentation. Humans have haemochorial placentas compared to the epitheliochorial placentas of the horse (reviewed in Wooding and Flint, 1994). The main differences between the two systems being the amount of invasiveness at implantation of the fertilised egg and the

intimacy of maternal and foetal blood supplies. In the horse, the uterine epithelium and conceptus come into contact and placental development essentially involves a vast increase in area with no loss of any tissue layers between the foetal and maternal bloodstreams. However, human placentation involves the conceptus rapidly intruding through the uterine epithelium into the endometrium followed by considerable decidual development to reduce the distance between foetal and maternal blood microflows to 4μm compared to 5-10μm in the horse (reviewed in Wooding and Flint, 1994). Details of the actual process by which the human conceptus gains entry to the endometrium are not known but it is not unreasonable to assume some proteolytic mechanism may play a part. Humans with a high antiproteolytic screen would thus be less fertile as the chances of successful implantation of the conceptus would be reduced. Interestingly, rats have the same type of placenta and a similar plasma concentration of API to humans (1.7 and 1.3g/l respectively).

Hence the reason for the difference in antiproteolytic screen between humans and horses may be due to the differences in placentation with the increased susceptibility to pulmonary emphysema being the cost humans have to pay for successful reproduction and our present longevity.

APPENDIX 1

PREPARATION OF SOLUTIONS AND BUFFERS

Phosphate-Buffered Saline (PBS)

8g NaCl

0.2g KCl

1.15g Na_2HPO_4

0.2g KH_2PO_4

Dissolve in 1 litre de-ionised, distilled water and adjust pH to 7.2-7.3

ENE 2A and Equine API ELISA Coating Buffer

1.59g Na_2CO_3

2.93G $NaHCO_3$

Dissolve in de-ionised, distilled water and make up to 1 litre. Adjust pH to 9.6 (if necessary). Used to dilute and bind primary 'capture' antibody to ELISA plate wells.

Deglycosylation Buffer

100mM $NaHPO_4$

50mM EDTA

5% 2-mercaptoethanol

2.5% Triton-X

The solution is adjusted to pH 7.5 after the addition of the first two constituents

APPENDIX 2

TISSUE CULTURE MEDIA

Serum Free Culture Media

Serum free culture media consisted of the following mixture:

RPMI 1640 with 20mM HEPES, 0.85g/l sodium bicarbonate

2mM L-glutamine

Penicillin/Streptomycin solution (100 units/ml and 100 μg/ml respectively)

Screening of Foetal Calf Serum

Samples of foetal calf serum (FCS) should be obtained from various suppliers noting batch number, batch size, price and length of holding time. Prepare 15% FCS/RPMI 1640 medium (as above) with various FCS and sterility check. Cell lines to be tested are then diluted out to one cell per well of a 96 well plate and incubated at 37°C in an humidified incubator with 5% CO_2 and checked on day 5-7 for growth of cells, followed by feeding and rechecking in 3 days. Antibody production from hybridoma cell lines is assessed as in Chapter 3.2.3. Serum is selected on the basis of cell growth, antibody titre and price.

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PUBLICATIONS ARISING FROM THIS THESIS

Abstracts

M. P. Dagleish[1] (1996) Characterisation of Mouse Anti-Equine Alpha-1-Proteinase Inhibitor Monoclonal Antibodies. The Respiratory System in Health and Disease, *The Wellcome Trust Diamond Jubilee Year*. p 41.

M. P. Dagleish[2], E. M. Milne, C. L. Scudamore, P. M. Dixon, S. M. M^{C}Aleese and A. D. Pemberton. (1996) Characterisation Of Pulmonary And Plasma Serpins In The Horse Using Polyclonal And Monoclonal Antibodies Against Equine Alpha-1-Proteinase Inhibitor. Jubilee Scientific Meeting, *Association of Veterinary Teachers and Research Workers*. p 29.

M. P. Dagleish[1], E. M. Milne, C. L. Scudamore, P. M. Dixon, S. M. M^{C}Aleese and A. D. Pemberton. (1996) Characterisation Of Pulmonary And Plasma Serpins In The Horse Using Polyclonal And Monoclonal Antibodies Against Equine Alpha-1-Proteinase Inhibitor. (1996) *International Symposium on the Chemistry and Biology of SERPINS*. p 103.

M. P. Dagleish[2], A. D. Pemberton, S. M. M^{C}Aleese, E. M. Thornton, H. R. P. Miller and C. L. Scudamore. (1998) Improved Hepatic and Pancreatic Localisation of Equine Alpha-1-Proteinase Inhibitor. 52nd Scientific Meeting, *Association of Veterinary Teachers and Research Workers*. p 44.

[1]Poster presentation. [2]Oral presentation.

Refereed Journals

M. P. Dagleish, A. D. Pemberton, S. M. M^{C}Aleese, E. M. Thornton, H. R. P. Miller and C. L. Scudamore. (1998) Improved Hepatic and Pancreatic Localisation of the Equine Alpha-1-Proteinase Inhibitor Family of Serpins using an Antigen Enhancement Technique and a Monoclonal Antibody. *Research in Veterinary Science* In Press.